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SECTION MEETINGS

CLEVELAND	
Western Reserve University	December 11, 1936
NEW YORK	
New York Academy of Medicine	January 20, 1937
PACIFIC COAST	
University of California Medical School	December 9, 1936
SOUTHERN CALIFORNIA	
University of Southern California	December 29, 1936
WESTERN NEW YORK	
Syracuse University	December 19, 1936

9034

Effect of Saccharin and of Galactose on the Blood-Sugar.*

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Syllaba[1] reported the existence in man of a conditioned hepatic reflex by which a significant rise in the blood-sugar level is produced 5 to 15 minutes following ingestion of saccharin. According to his opinion, based on the study of 9 cases, this reflex is released partly by contact of the solution of saccharin with the walls of the small

*Work on this problem was assisted by a grant from the Christine Breon Fund for Medical Research.

1 Syllaba, G., *Guy's Hosp. Rep.*, 1930, **80**, 230.

intestine, and partly by a psychic reaction depending on the sweet taste of saccharin. More recently Pannhorst[2] reported observations on reflex hyperglycemia elicited by rinsing the mouth with a solution of saccharin.

Blöch and Weiss[3] described a rise in the dextrose fraction of the blood, beginning 5 minutes after administration of 40 gm. of galactose to 9 patients with various diseases. They ascribed the early part of this rise to release of sugar from the liver. Harding and Grant[4] obtained similar results in most of their subjects, but made no determinations at the 5-minute period. The last-named authors raise the question of galactose-dextrose conversion versus release of sugar from the liver as an explanation of the rise in the dextrose curve.

Twenty normal individuals were directed to drink slowly on the fasting stomach, 200 cc. of water containing 0.15 gm. of saccharin. This amount was chosen as approximating most nearly the taste of the solution used for dextrose tolerance tests. Specimens of blood were taken from a finger for blood-sugar determinations before and at intervals of 5, 15, and 30 minutes after the ingestion of saccharin. Capillary blood was chosen in order not to overlook minor additions of sugar from the liver which might have become obliterated during passage through the muscles if venous blood had been examined.

Forty gm. of galactose were dissolved in 400 cc. of water, flavored with lemon juice and administered by mouth on the fasting stomach. Specimens of blood were obtained from the cubital vein before, and 5, 15, and 30 minutes after, administration of galactose. The dextrose and galactose fractions of these blood samples were determined separately by Raymond and Blanco's[5] modification of the method of Somogyi.

This experiment was performed on 21 normal individuals and on 14 patients with diabetes.

From the chart it is seen that after ingestion of saccharin there was no rise, but if anything a slight fall in the average blood-sugar of our normal subjects.

Following administration of galactose, a small rise in the average dextrose fraction of the blood was observed in normal individuals at the 15- and 30-minute periods. In our diabetic patients a considerable elevation of the dextrose fraction was seen, beginning 15

2 Pannhorst, R., *Z. f. klin. Med.*, 1935, **127**, 688.

3 Blöch, J., and Weiss, M., *Z. f. d. ges. exp. Med.*, 1929, **69**, 453.

4 Harding, V. J., and Grant, G. A., *J. Biol. Chem.*, 1933, **99**, 629.

5 Raymond, A. L., and Blanco, J. G., *J. Biol. Chem.*, 1928, **79**, 649.

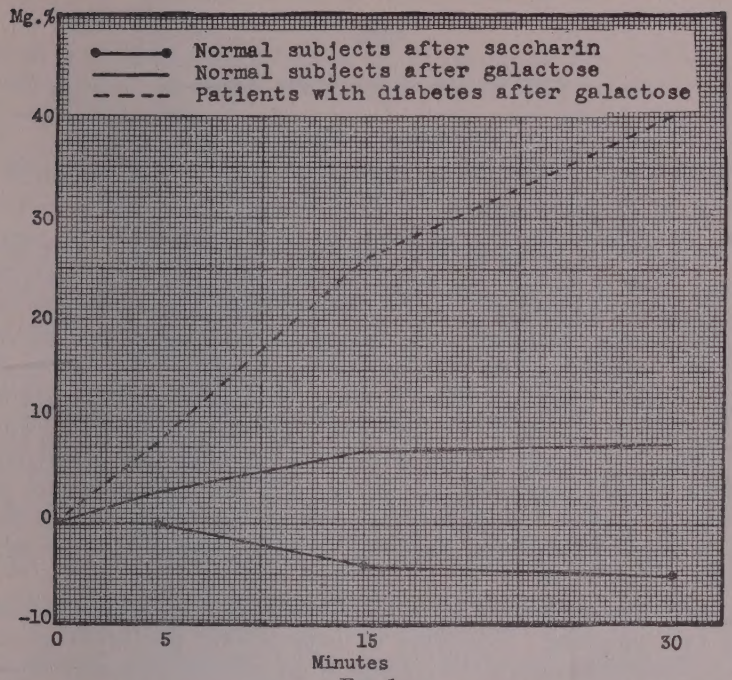

FIG. 1.

Chart showing composite changes in the total blood-sugar of 20 normal subjects after saccharin; and in the dextrose fraction of the blood of 21 normal individuals and 14 diabetic patients after galactose.

minutes after ingestion of galactose. Among 14 patients with diabetes, only one failed to show this.

In common with Roe and Schwarzman[6] we ascribe this elevation of blood-dextrose to failure on the part of the diabetic organism to utilize dextrose formed by conversion of galactose. For this reason, the small rise in the dextrose fraction of the blood observed in normal individuals, if significant at all, must be likewise due to conversion of galactose. Our observation that only a few normal and diabetic subjects showed small amounts of galactose in the blood at the 5-minute period, while practically all had considerable amounts of this sugar in the blood at the 15-minute period, supports this view.

Our data on the galactose fraction of the blood, to be published later, confirm the finding of Roe and Schwarzman that tolerance to galactose is not disturbed in diabetes mellitus.

Conclusion. Following oral administration of either saccharin or galactose, no evidence was obtained of a reflex hyperglycemia.

[6] Roe, J. H., and Schwarzman, A. S., *J. Biol. Chem.*, 1932, **96**, 717.

9035

Purification of Botulinus Toxin.

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It was shown[1] that a purified botulinus toxin of fairly high potency (MLD = 10^{-7} gm. per mouse) may be prepared by simple acid precipitation (precipitate I) of filtered culture fluid. Samples of 10-fold toxicity, however, were obtained by dialysis of the filtered acidified mother liquors. Under these conditions a fine sediment (precipitate II) appears which contains practically all the remaining toxicity of the solution and shows a potency of about 10^{-8} gm. per mouse. It is evident from these facts that some diffusible components of the culture fluid are able to hold the toxic substance in solution at a pH of about 4. The high amino acid content of the tryptic digest broths was suspected of being responsible for this effect.

In more recent experiments a peptic digest medium was therefore used as a substrate. According to expectations the toxin could now be precipitated almost completely by the addition of hydrochloric acid without dialysis. This latter somewhat cumbersome procedure can thus be entirely eliminated. The crude acid precipitable material possesses the same high potency of 10^{-8} gm. as found previously. The yield amounts to roughly 50%; very little poison is left in the filtrate. The precipitate may be further purified by dissolution in sodium acetate buffer and reprecipitated with n/10 hydrochloric acid. The most potent product obtained in this manner showed a toxicity of 4.10^{-9} gm. = 2.10^{-7} gm. per kg. mouse. The yield of the reprecipitated product has been variable and not large. It is possible, however, to avoid great losses by taking into consideration that botulinus toxin gradually deteriorates at room temperature and that the rate of inactivation is enhanced by the presence of trivalent anions.[2] Furthermore the pH has to be adjusted carefully to the flocculation point of the protein (pH 4.4) in order to avoid loss of potency in the opalescent supernatant.

This procedure, then, may be recommended for the preparation of relatively large amounts of highly potent botulinus toxin. It makes use of the most simple equipment and reagents. The medium before as well as after growth is acidified in carboy lots. Separation

[1] Snipe, P. T., and Sommer, H., *J. Infect. Dis.*, 1928, **43**, 152.

[2] Sommer, E. W., and Sommer, H., *J. Infect. Dis.*, 1928, **43**, 496.

from the sediment is performed by syphoning at first, and later by centrifuging. It is not necessary to remove the organisms before precipitation, since the bacteria, bacterial proteins and toxin are thrown out of solution in one operation at a pH of 3.5-4.0. After redissolution in sodium acetate the organisms may be removed by centrifuging and filtering through a Seitz filter. The volume, by this time, is small enough to be conveniently handled in liter bottles and centrifuge tubes.

It is reasonable to assume that this method should not be readily applicable to the purification of the acid-sensitive diphtheria toxin, as Eaton[3] points out, although Locke and Main[4] have prepared substances of high titer by fractional acid precipitation. On the other hand there is no apparent reason why potent tetanus toxin should not be obtained in the same manner. In fact, in one experiment performed in 1928, a tetanus toxin was obtained from tryptic digest broth by the dialysis procedure, killing mice in amounts of approximately 2.5×10^{-8} gm. This compares favorably with substances (MLD $= 3.8 \times 10^{-8}$) made recently by Eaton[5] by the more complicated procedure of precipitation with metal salts.

In conclusion it must be emphasized that these highly potent protein preparations should not be considered pure substances. The following fact illustrates this point. A sample of botulinus "toxin" prepared from washed and dried organisms by dissolution in phosphate buffer showed a potency of 10^{-8} gm. of dried organisms per mouse. Since this crude bacterial product was not much less toxic than the purest substance obtained by the above procedure, the chemically pure poison must be assumed to have a potency many times greater.

Summary. A simplified purification procedure, based on acid precipitation, is described for the preparation of highly potent botulinus toxin.

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[3] Eaton, M. D., *J. Bact.*, 1936, **31**, 347.

[4] Locke, A., and Main, E. R., *J. Infect. Dis.*, 1928, **43**, 41.

[5] Eaton, M. D., PROC. SOC. EXP. BIOL. AND MED., 1936, **35**, 16.

9036 P

Effects of Oxygen Tension and Temperature Change on the Bio-Electric Potential of Halicystis.*

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Previous experiments (external application of sap, perfusion of vacuole with sea water[1, 2]) have indicated that the large bio-electric potential (P.D. = 70 to 80 mv. outside +) across the protoplasm of impaled *Halicystis* cells (coenocytic marine alga) is mostly independent of inorganic ions or other gradients between cell sap and environment. Internal gradients or assymetries in the protoplasmic film itself were suggested. Since the P.D. can produce a measurable current for long periods through a completed circuit, a source of energy, eventually metabolic, must be available. Experiments have therefore been directed toward analyzing the relation between P.D. and metabolism in this organism. The electrical methods are those previously employed,[1, 2] the cells being kept in the dark to avoid photosynthesis. Manometric measurements of O_2 consumption by the Warburg method were made on groups of several intact cells under comparable conditions (except for impalement). Concurrent P.D. and respiration measurements on identical impaled cells are now under way.

Oxygen tension. Over the range where respiration is unchanged, the P.D. is also unaltered. This holds for pure O_2, air, and for mixtures of O_2 with N_2 down to about 2% O_2, brought into equilibrium with sea water by continued bubbling. If bubbling of the 2% O_2 mixture is stopped, the P.D. drifts downward as O_2 is consumed in the vicinity of the cell, eventually falling to 10 or 15 mv. +. Stirring of the sea water, or rebubbling of 2% O_2 promptly restores the P.D. This downward drift and recovery may be repeated almost indefinitely. Further lowering of O_2 tension, *e. g.*, to 0.2% O_2 in N_2, reduces the P.D. to 10 mv. or less while bubbling or stirring continues. Respiration also falls to low values in this mixture. Recovery of P.D. again occurs if 2% or higher O_2 is bubbled, unless the cells have been kept for many hours at low O_2 tensions. Curious downward cusps of P.D. often precede the recovery curve. In this respect, as well as in the general linkage of P.D.

* Aided by a grant from the Rockefeller Foundation.

1 Blinks, L. R., *J. Gen. Physiol.*, 1932, **16**, 147.

2 Blinks, L. R., *J. Gen. Physiol.*, 1935, **18**, 409.

with respiration, these findings agree with those of Lund,[3] Francis,[4] and others on other organisms. The exact nature of the linkage is not demonstrated however; respiration may be necessary to maintain either surfaces or other structures, or a supply of organic ions. An O_2 gradient, *as such* across the protoplasm is not responsible, for the P.D. is equally well restored, in its original sign, by perfusion of aerated sea water through the vacuole while 0.2% O_2 is bubbled outside.

Temperature, of course, markedly influences respiration, which has the usual Q_{10} of 2.0 or more in the temperature intervals 5° to 15° (normal), and 15° to 25°C. (in intact *Halicystis* cells; preliminary work with impaled cells shows similar values). The P.D., however, is lowered only 10 or 15% at most when the cells are cooled from 15° to 5°, even for several days ($Q_{10} = 1.1$ to 1.2). There is still less increase of P.D. when cells are warmed from 15° to 25° or higher ($Q_{10} = 1.1$ or less). Relatively small temperature effect on P.D. was also found by Hill in *Nitella.*[5]

These small temperature effects may indicate that some physical process such as diffusion limits the P.D., the supply of ions being more than sufficient to maintain the gradients. Independent evidence,[6] however, indicates that in *Halicystis* 2 large discrete potentials, + and —, summate to give the observed P.D. across the protoplasm. If these had large but slightly different individual Q_{10} values, their algebraic sum might remain nearly unchanged with temperature. The somewhat larger cusps obtained on the sudden immersion of cells in warmer or cooler sea water might represent the momentary lagging of the inner surface behind the outer while a radial temperature gradient existed. Larger temperature effects in other organisms[7, 8] might represent the lessening or absence of such summation, or very different Q_{10} values for the component potentials.

Under altered conditions, large temperature effects may also be obtained in *Halicystis.* Thus the downward drift of P.D. after bubbling of 2% O_2 is stopped, occurs only very slowly or not at all at 5°C., probably because diffusion can now supply O_2 as fast as it is respired. Warming to 15° then causes it to fall, while at 25°

[3] Lund, E. J., *J. Exp. Zool.,* 1928, **51**, 265.

[4] Francis, W. L., *J. Exp. Biol.,* 1934, **11**, 35.

[5] Hill, S. E., *J. Gen. Physiol.,* 1935, **18**, 357.

[6] Blinks, L. R., Rhodes, R. D., and McCallum, G. A., *Proc. Nat. Acad. Sci.,* 1935, **21**, 123.

[7] Umrath, K., *Protoplasma,* 1934, **21**, 329.

[8] Marsh, G., *Carnegie Inst., Washington,* 1936, Pub. No. 475. 1.

it may even fall during bubbling or stirring. A large apparent Q_{10} results, but with negative sign; a higher respiratory rate correlates with a lower P.D. and *vice versa*.

Other conditions facilitate large temperature effects. 5 to 10% CO_2, mixed with air and bubbled through sea water, depresses the P.D. of *Halicystis*, probably by raising the protoplasmic acidity. The CO_2 tension at which this occurs is a function of temperature. A similar situation holds for dilute ammonia, which causes reversal of P.D.[9] At 15° the threshold for this lies around 0.002 M NH_4Cl in sea water; the threshold concentration is increased by cooling the sea water, and decreased by warming (probably by altering the dissociation and solubility of ammonia). As a result, a 10° change may alter the P.D. by 100 to 150 mv., giving very large apparent Q_{10} values. The sign is negative, warming causing more negative values, cooling more positive ones (*cf.*, *Valonia*[8]). Temperature also facilitates the reversal of P.D. by vacuolar perfusion of alkaline sea water[2]; this occurs in *H. ovalis* at 25° but not at 15°. Possibly other large temperature effects are similarly conditioned by physiological alterations of O_2, CO_2, ammonia, and pH.

Time curves of these effects, as well as those of other agents influencing metabolism and P.D., will be reported elsewhere.

9037 P

Zinc Sulphate as a Chemoprophylactic Agent in Experimental Poliomyelitis.*

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In earlier notes[1] we reported observations on the prophylactic action exercised by picric acid and certain other compounds. In this note we are reporting results which indicate that zinc sulphate applied to the nasal mucosa is even more effective than picric acid. These observations are set forth in Table I, in which the relative efficiency of the respective agents is compared in terms of the ap-

[9] Blinks, L. R., *J. Gen. Physiol.*, 1933, **17**, 109.

* Studies supported by funds from the President's Birthday Ball Commission for Infantile Paralysis Research.

[1] Schultz, E. W., and Gebhardt, L. P., PROC. SOC. EXP. BIOL. AND MED., 1936, **34**, 133; *Calif. and West. Med.*, 1936, **45**, 38.

TABLE I.

Exp.	Type of Experiment	No. of monkeys treated	% treated animals protected 1 mo.	No. of controls	% infection
1	1% picric acid; 3 intranasal irrigations; successive days; virus (a)	16	62	30	80
2	1% picric acid; 3 intranasal sprays*; virus (b)	30	37	20	100
3	1% picric acid; 6 sprays*; virus(b)	10	25	12	100
4	.5% picric acid; 6 sprays*; virus(b)	8	12		
5	.5% picric acid plus .5% alum; 6 sprays*; virus (b)	19	71	10	100
6	.5% picric acid plus .5% zinc sulphate; 6 sprays*; virus (b)	20	86	21	100
7	.5% zinc sulphate; 6 sprays*; virus (b)	9	100		
8	1% zinc sulphate; 5 sprays†; virus (c)	5	100	22	90
9	1% zinc sulphate; 4 sprays†; virus (c)	5	100		
10	1% zinc sulphate; 3 sprays†; virus (c)	12	92		
11	1% zinc sulphate; 2 sprays†; virus (c)	5	80		
12	1% zinc sulphate; 1 spray†; virus (c)	3	100		
13	1% zinc sulphate; 3 intranasal irrigations successive days; virus (c)	14	100		

*The chemical solution was applied to the nasal mucosa on successive days as indicated, and once a week thereafter. Applications were made with a de Vilbiss atomizer attached to nitrogen tank and under 6 lbs. pressure. Solution warmed to 38°C.

†Same as described under*, but not "once a week" after the initial daily applications indicated.

(a) Virus instilled intranasally 3 times on same day, each instillation preceded by acid phosphate lavage. Instillations 2 to 6 days and again 4 weeks after application of chemicals.

(b) Virus instilled intranasally 5 successive days each week for 3 to 4 weeks.

(c) Virus instilled intranasally on 5 successive days beginning one month after chemical was applied to mucosa; no preliminary washes with acid phosphate solution.

proximate per cent of animals which were protected for at least one month against repeated intranasal instillations of poliomyelitis virus.

While the number of animals in individual experiments and the mode of treatment and the intensity of the subsequent virus inoculations are not all strictly comparable, certain experiments are sufficiently alike to reveal important differences in the degree of protection. For example, in Experiment 4, in which 8 monkeys were treated with 0.5% picric, and in Experiment 7, in which 9 monkeys were treated with 0.5% zinc sulphate, but otherwise were dealt with in the same way, there is a difference amounting to 88% in favor of $ZnSO_4$. Furthermore, the addition of zinc sulphate to picric acid (Experiment 6) enhanced its protective value somewhat

more than did the addition of alum (Experiment 5). Of significance also is the fact that zinc sulphate when used alone has afforded over 95% protection in a total of 53 animals (Experiments 7 to 13). Thus far, only 2 animals treated with zinc sulphate alone have succumbed to poliomyelitis. Six animals have survived repeated virus instillations for 3 months.

It will be noted that the protection afforded by picric acid is distinctly greater in Experiment 1 than in Experiments 2, 3, and 4. This is accounted for by the fact that in the latter experiments, virus was instilled intranasally almost daily during the entire month; an exposure which brought down 100% of the controls. Even under these drastic conditions, none of the 9 monkeys treated with 0.5% $ZnSO_4$ (Experiment 7) developed the disease.

Studies are in progress to determine the lowest dilutions of zinc sulphate which will protect monkeys for at least one month and to determine the factors which influence the effectiveness and duration of the protection.

In presenting these observations, we do not wish to imply that any of the agents described thus far will necessarily prove effective in the prevention of poliomyelitis in man. The results, however, lay a foundation for similar studies in man during an epidemic period.

9038 P

Complement Fixation Test Differentiating 3 Strains of Equine Encephalomyelitic Virus and the Virus of Lymphocytic Choriomeningitis.

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Although most of the attempts to apply the complement fixation test in the field of filterable viruses have either been negative or inconclusive, within recent years a few favorable reports have lent more encouragement. The work of Craigie[1] and his coworkers has been of especial value in suggesting a new method of attack. Based on their reports, work was undertaken to extend the test for the differentiation of strains of equine encephalomyelitic virus, representing the so-called eastern and western American types and that

[1] Craigie, J., and Tulloch, W. J., *Sp. Rep. Ser. Med. Res. Coun. London*, No. 156, 1931; Craigie, J., and Wishart, F. O., *Can. Pub. Health J.*, 1936, **27**, 371.

of Moscow No. 2. All 3 of the strains may be differentiated serologically by the neutralization test.[2] The virus of lymphocytic choriomeningitis isolated by Dr. C. Armstrong of the National Institute of Health, was also included in the work.

With some slight modifications the antigens were prepared according to the method of Craigie and Tulloch[1] for vaccinia. The equine viruses were carried in the guinea pig and that of the "l.c.m." virus in the mouse. The brains were removed aseptically from the respective infected animals, were frozen in an ice-salt mixture and dried in a desiccator evacuated by the Cenco-Hyvac pump. The dried material was weighed and ground with ether in the desired proportions. The mixture was shaken, stored on ice for several hours. Then the ether was removed; the residue was ground with 0.85% saline, left for 6-12 hours on ice, frozen several times in an ice-salt mixture and thawed at 37°C. Finally the material was centrifugated and the supernatant fluid removed, which was then ready for use after a preliminary titration for anticomplementary, hemolytic and antigenic properties.

After a careful titration of the complement with the antigen, the test was set up with 0.1 cc. of serum from guinea pigs hyperimmunized to each strain. 0.2 cc. of antigen and 0.2 cc. of complement containing 2 full hemolytic units were then added and the mixture left for 16 hours at refrigerator temperature. Five per cent washed sheep cells sensitized with 2 units of hemolysin (final dilution 2.5% RBC) were added and the tubes left for 15 to 20 minutes in the water bath at 37°C. or until all the control tubes except that for the sheep cells showed complete hemolysis. Readings were made immediately and again some time later.

Cross immunization tests were performed with immune serums from each strain used. The various serums were also tested against antigens prepared in a similar manner from rabbit brain containing the Borna type of European equine encephalomyelitic virus, with normal guinea pig brain and with mouse brain containing the virus of lymphocytic choriomeningitis of Armstrong and Lillie.[3] Positive fixation occurred between hyperimmune serums and the homologous antigens of each type of virus but not between the same serums and heterologous antigens; it was not observed in the presence of normal guinea pig brain or Borna antigens. Specific fixation took place between immune serums and the homologous antigen of the "l.c.m." virus but there was no cross reaction whatever between this virus and

[2] Howitt, B. F., *J. Immunol.*, 1935, **29**, 319.

[3] Armstrong, C., and Lillie, R. D., *Pub. Health Rep.*, 1934, **49**, 1019.

those of the equine strains. No immune serum was available for the Borna virus. It was noticed that while not all immunized guinea pigs responded by a marked antibody formation the serum of certain animals consistently gave positive results over varying periods of time with antigens made on different occasions. This was particularly true for serums of the Moscow No. 2 strain.

Two series of animals were immunized with live virus of the eastern and western American and the Moscow No. 2 strains, respectively, over a period of several months. Blood was removed from the heart and increasing doses of virus were administered at weekly intervals. One series was given intravenous and the other subcutaneous injections of virus, respectively. Neutralization and complement fixation tests were performed on serums collected weekly with results indicating a parallelism between the 2 reactions, although the neutralization test usually became positive at an earlier period than the complement fixation. As a rule the latter failed to take place with serums of the American equine strains until after 2 months of immunization and then was not strongly positive until massive doses of virus had been given. The animals inoculated with the Russian strain gave the strongest reaction in the shortest time.

From the results it seems apparent that the complement fixation test may be applied to the differentiation of the strains of equine encephalomyelitic virus and to that of the lymphocytic choriomeningitis virus of Armstrong and Lillie. So far the test seems specific for the homologous strains when strongly hyperimmunized serums are used together with a potent antigen. Further work is contemplated in this field with other viruses and a more comprehensive account of the results already obtained will be reported later.

9039

Total Nitrogen Content of Skeletal Muscle of the Rat in Various Nutritional States.

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It is generally conceded that one important function of the growth hormone of the anterior hypophysis is the promotion of protein anabolism. Evidence for this has been obtained from the results of numerous nitrogen balance studies which have been published in

recent years, all of which show a marked retention of nitrogen after single or multiple doses of the hormone. The same conclusion has been drawn from several studies of the nitrogen content of animals, in which it has been found that the tissue nitrogen is significantly higher after growth hormone has been administered. So far as we are aware, however, all of these analyses have been made on the entire carcass, the soft tissues, or on the liver. With the exception of liver, therefore, no information is available on the influence of growth hormone on the nitrogen content of individual organs or tissues. It seemed, then, to be of interest to include analyses of skeletal muscles in the course of a general study of the effect of various nutritional conditions on growth and development in rats.

The rats were of standard, pedigreed stock, and were placed on the special diets when 13 weeks old. Seven groups of 7 to 10 rats each were treated as follows:

Group 1. Control, on normal diet only.*

Group 2. High phosphorus diet: disodium phosphate was added to the normal diet in sufficient quantity to increase the phosphorus content to 1.25%-1.5%.

Group 3. Normal diet with addition of 22.5 units of vitamin B_1 per day in the form of Tiki-tiki extract† during the first 10 weeks, thereafter 45 units per day.

Group 4. High phosphorus diet with the same vitamin B_1 supplement as in group 3.

Group 5. Normal diet and growth hormone (Antuitrin Growth‡) given intraperitoneally.

Amount of hormone administered: First 2 weeks, 8 units per week in 4 equal doses. Next 6 weeks, 4 units per week in 4 equal doses. Remainder, 8 units per week in 4 equal doses.

Group 6. High phosphorus diet and growth hormone administered as in group 5.

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* The stock diet used was:

	%
Whole wheat flour	68.3
Crude casein	13.8
Whole milk powder	9.2
Sweet butter	6.4
Calcium carbonate	1.36
Sodium chloride	0.91

The calcium and phosphorus content of this diet varied between 0.5% and 0.6%. The protein content was approximately 23%, and the calorific value varied from 4.8 to 5.0 large calories per gram. These values were not significantly varied in the other diets.

† Supplied by Harris Vitamin Laboratories.

‡ Supplied by Parke Davis and Company.

TABLE I.

Results of Analyses on Quadriceps Muscle in Rats.

Group	Water				Nitrogen (Wet basis)				Nitrogen (Dry basis)			
	No. of analyses	Mean H_2O content, %	Std. dev.	Std. error	No. of analyses	Mean N_2 content, %	Std. dev.	Std. error	No. of analyses	Mean N_2 content, %	Std. dev.	Std. error
1	6	74.60	.27	.12	6	3.547	.066	.027	6	13.93	.334	.126
2	6	74.90	.46	.19	7	3.456	.069	.026	7	13.63	.198	.075
3	6	74.81	.14	.06	7	3.485	.053	.020	7	13.85	.166	.063
4	8	74.90	.48	.17	9	3.506	.063	.021	9	13.90	.269	.089
5	9	75.43	.53	.19	9	3.488	.059	.021	8	14.18	.142	.054
6	5	74.81	.31	.14	5	3.477	.060	.022	5	13.80	.145	.065
7	9	74.64	.63	.21	9	3.434	.044	.015	9	13.62	.322	.107

Group 7. Diet of group 4, with the addition of vitamin D§ in the form of viosterol of such a concentration that the volume of oil did not exceed 0.125 cc. daily per rat, for a total weekly supplement of 12.5 units of the vitamin.

The various diets were fed for 7 months without interruption; the animals being 10 months old when killed for analysis. All animals appeared to be healthy and vigorous when killed, with the exception of certain animals which had previously developed an infection of the labyrinths. The values found on analyses of the infected animals were the same as for the others, and are included in the results in Table I. Values are expressed in per cent. The effect of the growth hormone on the weight of the rats was not as marked as might have been the case if the administration had been started earlier. Rats receiving the hormone were, however, heavier than the controls. It was noted also that males in group 7 were heavier than the controls, while the females were lighter. The vitamin B_1 supplement in groups 3 and 4 apparently had no significant effect on growth or health of the animals.

The nitrogen determinations were made in duplicate by macro Kjeldahl technique on the quadriceps muscle. The animals were anesthetized with ether, the muscles excised and placed in solid carbon dioxide. A part was used for determination of water content, and the remainder for nitrogen determination. The results of the analyses are shown in Table I.

The probabilities of the results shown in Table I being random have been calculated by the statistical method of Fisher,[1] which takes into account the population of the series. From these it can be said that the water content of the muscles in group 5 was significantly higher than in any other group, with the possible exceptions of groups 2 and 6, and in group 6 the series is short. This is the more interesting when it is remembered that groups 5 and 6 received equal amounts of growth hormone, the only difference being the phosphorus content of the diet.

The nitrogen content of the moist muscles of group 7 was significantly less than all others except group 2. Group 2 contained less nitrogen than group 1. All the others were well within the experimental error.

When the nitrogen content is calculated as per cent of dry weight, the various groups show a more complex relationship. Group 5 contained significantly more nitrogen than all other groups, in-

§ Supplied by Mead Johnson and Company.

[1] Fisher, R. A., *Statistical Methods for Research Workers*, Second Edition, Oliver and Boyd, Edinburgh, 1928.

cluding group 6. Group 2 contained less than groups 1, 3, 4, and 5, while group 7 contained less than groups 1, 4, and 5.

It seems clear from these results that on a normal diet growth hormone increases the nitrogen content of skeletal muscle while at the same time increasing the water content. The effect of the other factors studied, vitamins B_1 and D and excess phosphorus, is less obvious and further studies are in progress on this phase of the problem.

Summary. 1. The total nitrogen content of the quadriceps muscle of the rat in various nutritional states has been studied. 2. It has been found that, on a normal diet, growth hormone increases both the total nitrogen and the water content of the muscle.

9040

Effects of Prolonged Administration of Moderate Doses of Creatine in Rats.

H. C. STRUCK AND M. B. VISSCHER.

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Since there were no observations reported in the literature on the long continued administration of creatine to experimental animals, it seemed worth while to conduct a series of observations on young and old animals in which creatine was fed in doses comparable to those used in certain clinical therapeutic experiments in man. This seemed especially desirable since unfavorable effects of long-time administration might conceivably occur. The series of experiments was further used in order to determine the influence of moderately high ingestion rates of creatine on the amount of that substance stored in various tissues in the body, particularly in muscle. Rats were, therefore, fed on the following stock diet, half of the animals receiving this diet alone, and the other half receiving the same food mixture with 2 gm. of creatine hydrate added to each kg. of food. Assuming an average food intake of 14 gm. per day per rat of 350 gm. weight, the creatine intake was 75 mg. creatine hydrate per kg. per day.

The rats were from 2 sources. One group consisted of 25 animals* age 3 years or over at the beginning of the experiment,

*We are indebted to Professor Lloyd Arnold for providing these animals.

STOCK DIET

Sweet butter fat	6.5%
Whole wheat flour	68.2%
Whole milk powder	9.2%
Crude casein	13.8%
NaCl	0.9%
$CaCO_3$	1.4%
	100.0

In addition lettuce was fed to all rats twice weekly.

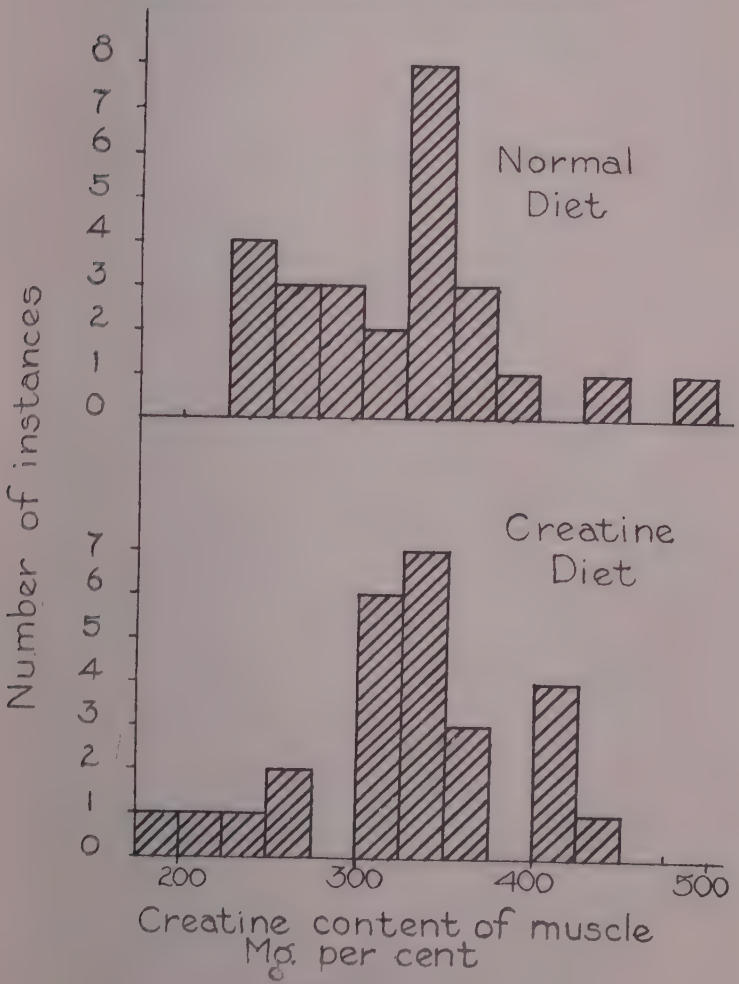

FIG. 1.

which had been obtained from the breeding colony of the Wistar Institute. The other group was of 50 Wistar strain animals 15 months of age at the beginning of the experiment. One-half of the animals in each group were placed on the control and the experimental diets for a period of from 4-6 months, at which time those still living were sacrificed and analyses made. The animals were anesthetized with amytal, 50 mg. per kilo, intraperitoneally. The tissues analyzed were the gastrocnemius muscle, the liver, and heart, which were excised in that order and plunged immediately into liquid air on excision. The tissues were weighed, ground with sand in a mortar containing trichloracetic acid after the method of Eggleton.[1] The creatine was estimated by the method of Folin-Benedict.[2]

TABLE I.
Creatine Content of Various Tissues of Rats.

	Normal diet			Creatine diet		
	Mean creatine content, mg./100g.	Std. dev.	No. of analyses	Mean creatine content, mg./100g.	Std. dev.	No. of analyses
Muscle	321.	58.	26	373.	69.	26
Liver	5.8	0.8	12	6.3	0.8	12
Heart	161.	56.	20	143.	43.	21

The distribution of creatine content in all of the analyses made on striated muscle is indicated in Fig. 1. It will be apparent that the most frequent concentration was the same in the animals on the stock diet as in those with creatine added. The mean values found in muscle, liver and heart, together with the statistically evaluated standard deviation for each, is shown in Table I. It is apparent that the means of the creatine content for the normal and the creatine series in no case differ by more than the standard deviation. Whatever differences exist are, therefore, probably those of random sampling. This seems the more likely because the direction of change is opposite for striated muscle and for cardiac muscle.

Particularly in the case of the rats 3 years of age and older a number of the animals died before the end of the experiment. Analyses were not made on those animals that died during the experimental period. Deaths in both the control and the experimental series of animals were due to a variety of causes, such as infections of the urinary tract, tumors, etc., which are common in animals of the age in question. There was no evidence that the animals on

[1] Eggleton, G. P., and Eggleton, P., *J. Physiol.*, 1929, **68**, 193.
[2] Benedict, S. R., *J. Biol. Chem.*, 1914, **18**, 191.

the creatine diet died as a result of specific poisoning. The analytical values for the young adult and the old animals showed no significant difference attributable to age. The figures have, therefore, been combined in the results presented.

It has previously been found (Chaikoff[1]) that massive doses of creatine over shorter periods may increase the content of creatine in rats. Our results indicate that more prolonged feeding of lower doses, which in total exceed the quantities fed over the shorter periods, does not lead to an augmentation in creatine content of the tissues we have examined.

9041

Are Neutral Fat and Lecithin Present in Gall Bladder Bile?

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Since the time of Strecker[1] chemists who have worked with bile have appreciated the difficulty of separating soaps from fat. The lipoid constituents as given by the text books at the present time are merely repetitions of the early figures given by Strecker, Hammarsten,[2] von Gorup Besanez[3] and others. These constituents are given as fatty acids, soaps, phosphatides, fat and cholesterol. An examination of this early literature does not yield satisfactory evidence for the presence of either neutral fat or lecithin in bile.

Fresh bile may be extracted in 2 ways. One is to deproteinize by means of alcohol, then dry the protein-free bile and dissolve the residue in absolute alcohol, filtering off the inorganic salts that are precipitated, and finally pouring the alcoholic solution into a large volume of anhydrous ethyl ether. This is the method used by Hammarsten.

The second method is to extract directly with ethyl and petroleum ether in the presence of some alcohol. In this manner a very persistent emulsion is formed that requires time to break. In either

[1] Chaikoff, A., and Beard, H. H., *J. Biol. Chem.*, 1928, **78**, 187; Chaikoff, A., and Silvette, H., *J. Biol. Chem.*, 1928, **80**, 589; Chaikoff, Alfred, *J. Biol. Chem.*, 1930, **89**, 705.

[1] Strecker, *Compt. Rend.*, 1851, **52**, 1249.

[2] Hammarsten, *Nova Acta Reg. Soc. Upsala*, 1898, June 15.

[3] Von Gorup Besanez, *Prager Vrtljahrsschrift für prakt. Pharm.*, 1851, **III**, 86.

method a sticky molasses-like substance is obtained on evaporating the ether, which does not look like fat and contains bile salts, fatty acid, phosphorus, sulphur and nitrogen containing compounds and non-saponifiable material. After as thorough an extraction as can be carried out in this manner, saponification of the non-ether soluble residue with strong alkali will permit the extraction of much fatty acid that could not be extracted in the first place.

When 2,500 cc. of beef gall bladder bile is dried in air, and then taken up in alcohol and precipitated in ether, the ether takes up 11.45 gm. of material or 0.458%.

Suspecting that the drying of bile and solution in alcohol might hydrolyze the fats, bile was extracted directly using a modification of the Roese-Gottlieb[4] method for fat extraction from milk. This method proved very difficult on account of the persistent emulsions that were formed. A typical experiment of this kind will be described. To 800 cc. of fresh hog bile placed in a tall cylinder, were added 200 cc. of alcohol, and 1,000 cc. of a mixture of equal parts by volume of ethyl and petroleum ether. The cylinder was shaken vigorously and then allowed to stand for several days. In time this mixture separated into 4 layers. On the bottom was a yellow precipitate of protein nature, above this a clear brownish colored watery solution of bile, above this a cloudy gray emulsion of bile and ether in which was suspended some of the protein precipitate, and finally above this a layer of clear ether. Both the clear layer and the emulsion were removed separately. The clear watery solution of bile was again extracted 3 or 4 times in the same way. The clear ether extracts were united and the emulsions were combined and evaporated to dryness. The residue from this was redissolved in ether and filtered, when a clear solution was usually obtained. All the ether extracts were now united, evaporated to dryness and the fatty material recovered.

The ether-soluble residue from this extraction was a brown colored, waxy solid containing much soft crystalline material and was 0.48% of the original bile (480 mg. per 100 cc.) On saponification and reextraction of the material extractable in ether from an alkaline solution, 80% of the material was recovered as non-saponifiable matter and of this 78% was cholesterol by the Liebermann-Burchard method. The remainder was probably higher alcohols. Hence, from 100 parts of this fatty substance 20% was fatty acid, 62% cholesterol and 18% non-saponifiable matter other than cholesterol.

[4] Roese-Gottlieb, *J. Nahr. Genuss m.*, 1905, **9**, 581; *A. O. A. C. Methods*, 1925, 262.

On saponification of a sample of the whole bile by means of potassium hydroxide, followed by extraction with petroleum ether, the non-saponifiable matter was 2.2% and the saponifiable matter 2.82%. Hence the direct extraction of fatty material from bile yields only a very small part of the total fatty acids that are present in bile.

The mixed gall bladder bile of at least 30 different steers was subjected to this extraction. From 2100 cc. of this bile 10.95 gm. of material were obtained, of which one gram was non-saponifiable material. This 10.95 gm. of material was saponified with sodium hydroxide for 24 hours, cooled, acidified with hydrochloric acid and allowed to stand in the cold for 12 hours. The fatty acids were filtered off and the filter washed twice with cold water. This clear filtrate was treated with benzoyl chloride as in the Schotten-Baumann method for alcohol determination as described by Mulliken.[3] Thirty-five milligrams of precipitate was obtained that in no way resembled glyceryl mono- or tribenzoate and which gave no odor of acrolein when heated with potassium acid sulphate.

Under the above conditions as little as 12 mg. of glycerol in dilute solution can be easily identified by this method. By the acrolein method alone as little as 1 mg. can be detected. Hence in this fatty material there must be less than 50 mg. of neutral fat or lecithin or less than 0.5% of the possible amount.

The acrolein test was further made on fatty extracts from dog, beef, and hog bile in amounts of from one to 2 gm. of ether soluble extract. In no case could the odor of acrolein be detected.

On one large sample of fat (title) an attempt was made to separate glycerol by the formation of sodium glyceride.[4] This gave a negative result. On 2 trials with 100 gm. of dog bile it was attempted to isolate glycerol directly by extracting the fat, saponifying and acidifying this fat, and then removing all interfering substances from the watery solution. On evaporating this watery solution nothing that resulted like glycerol could be detected.

In conclusion, these experiments conducted on the mixed gall bladder bile of the ox, the hog, and the dog indicate that neutral fat and lecithin are either absent from the gall bladder bile of these animals or is present in very minute quantities.

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[3] Mulliken, *Identification of Pure Organic Compounds*, 1914, Vol. I, 149.

[4] Heinrich, *Bull. Trade Assn. Formen de Teroga*, 1917, **12**. Abstracted in *Chem. Abst.*, **1917, 11, 216.**

9042

Glycogen Formation After Alanine Administration in Adrenalectomized Animals.*

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According to Evans,[1] the metabolism of endogenous protein is interfered with in the adrenalectomized rat. In our present studies the metabolism of an orally administered amino acid, alanine, has been investigated in animals with the adrenal gland removed. Butts[2] has shown that this amino acid is converted to glycogen in the organism, the maximum storage being 8 hours after the oral administration of the acid.

To 7 normal male and a like number of normal female rats 1.6 mg. alanine per square centimeter of body surface, was administered by stomach tube. A like amount was given to the paired brothers and sisters of the above animals which had been bilaterally adrenalectomized 6 days previously and had received salt solution since. All animals were fasted 24 hours before the alanine was fed. Three animals of each sex and of each group were given an amount of water by stomach tube equivalent in volume to the alanine solution fed in the other group.

In the groups of normal and adrenalectomized males, blood sugars were determined on blood obtained from the tails just before alanine administration and at the periods after administration shown in the table. At the end of 8 hours the livers of all animals were removed under amytal anesthesia and glycogen determined in the organ. A summary of the results is given in Table I.

Apparently adrenalectomy does interfere with the conversion of alanine to glycogen. The blood sugar changes are also less marked. Not only this but all of the adrenalectomized animals showed a drop in the sugar level after the peak which was reached one hour after alanine administration, while the normal rat showed a high level throughout the 4-hour period. The failure to store glycogen may be due not to failure of glycogen synthesis but to low blood sugar level.

The difference between normal males and females in ability to

*This work was assisted by a grant from the American Medical Association.

1 Evans, Gerald, *Am. J. Physiol.*, 1935, **114**, 297.

2 Butts, J. S., Dunn, M. S., and Hallman, L. F., *J. Biol. Chem.*, 1935, **112**, 263.

TABLE I.

	No. of Animals	Blood Sugar Fast	½ hr.	1 hr.	2 hr.	3 hr.	4 hr.	Liver Glycogen % at 2 hr.	Alanine % recovered
Normal ♂; Fed Alanine	7	89	113	111	102	92	109	75	96.94
" " " H_2O	3							[illegible]	
Adrenalectomized ♂; Fed Alanine	7	85	88	98	93	93	88	.69	97.41
" " " H_2O	3							.16	
Normal ♀; Fed Alanine	7							.79	100.99
" " " H_2O	3							.11	
Adrenalectomized ♀; Fed Alanine	7							.24	99.21
" " " H_2O	3							.08	

store glycogen, as reported by Deuel and [illegible] after glucose administration, does not appear in this experiment. Further work is being continued to determine the course of alanine metabolism in adrenalectomized animals.

9043

Effect of Transfusion of Blood From Dogs with Experimental Renal Hypertension into Normal Dogs.*

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Ischemia of the dog's kidney produced by constriction of the renal arteries, results in a chronic hypertension (Goldblatt, Lynch, Hanzal and Summerville [1]). Destruction of the nerves to the kidney does not prevent the development of the hypertension (Page[2], Collins[3]). Hence it may be supposed that we are dealing with a chemical mechanism. A number of workers (Prinzmetal and Friedman[4]; Harrison, Blalock and Mason[5]; Govaerts and Dicker[6]) have

3 Deuel, H. J., Jr., Gulick, M., Greenwald, C. and Cutler, C. H. *J. Biol. Chem.*, **1934, 104**, 519.

* Aided by a grant from the research funds of Graduate School of University of Minnesota.

1 Goldblatt, H., Lynch, J., Hanzal, R. F. and Summerville, W. W. *J. Exp. Med.*, **1934, 59**, 347.

2 Page, I. H., *Am. J. Physiol.*, 1935, **112**, 166.

3 Collins, D. A., *Am. J. Physiol.*, 1936, **116** 616.

4 Prinzmetal, M., and Friedman, B., Proc. Soc. Exp. Biol. and Med., 1936, **35**, 122.

5 Harrison, T. R., Blalock, A., and Mason, M. F., Proc. Soc. Exp. Biol. and Med., **1936, 35**, 38.

6 Govaerts, P., and Dicker, E., *Compt. rend. Soc. de biol.* 1936, **122** 209.

reported that extracts from the kidneys of dogs with such experimental renal hypertension exert a greater pressor effect than similar extracts from normal kidneys. This finding leads one to believe that the hypertension is due to the liberation of pressor substances into the blood stream from the ischemic kidney. The theory would be greatly supported if increased amounts of pressor substance could be demonstrated in the blood. Dicker[7] in fact reports that the blood of such dogs contains a hypertensive substance, while that of normal dogs does not. However, Page[8] could not find increased pressor substances. Alcohol extracts of the plasma of such dogs, when injected into cats, showed no more pressor activity than similar extracts made from normal plasma. We have attempted in this investigation to demonstrate increased pressor substances in the blood by massive transfusions from large dogs with renal hypertension into small normal dogs. The results have been entirely negative.

The hypertension in the donor dogs was produced by constriction of the renal arteries by a method described previously (Collins[3]). The systolic blood pressures of these animals varied from 180 to 230 mm. Hg. These dogs were large (20-30 kg.), and small dogs (4.5-9 kg.) were chosen as recipients in order that large amounts of blood, in relation to the size of the animal, could be injected. For example in one experiment the recipient dog received an amount of blood equal to 20% of his weight. As the blood was injected, an equivalent amount was withdrawn from the animal through a cannula in the femoral artery, thus avoiding the effects of plethora. The bloods of the 2 animals were always previously checked for incompatibility by a cross agglutination test. This precaution is wise, as Ottenberg, Kaliski and Friedman[9] and also McEnery, Ivy and Pechous[10] have shown. The arterial blood pressure of the recipient dogs was recorded by means of a mercury manometer, connected to a carotid artery. Morphine-ether anesthesia was employed in all except one case, since it does not lower the blood pressure of dogs with experimental renal hypertension. Table I illustrates this fact.

The transfusions were accomplished in two ways. In the first method no anticoagulant was used. The blood was taken from the femoral artery of the donor either with a needle and syringe or by

[7] Dicker, E., *Compt. rend. Soc. de biol.*, 1936, **122**, 476.

[8] Page, I. H., PROC. SOC. EXP. BIOL. AND MED., 1936, **35**, 112.

[9] Ottenberg, R., Kaliski, D. J., and Friedman, S. S., *J. Med. Research*, 1913, **28**, 141.

[10] McEnery, E. T., Ivy, A. C., and Pechous, C. E., *Am. J. Physiol.*, 1924, **68**, 133.

TABLE I.

Time, min.	Arterial blood pressure, mm. Hg.	Time, min.	Arterial blood pressure, mm. Hg.	Time, min.	Arterial blood pressure mm. Hg.
		60	210/108	100	204/104
5	192/118	65	212/108	110	212/114
10	188/106	70	208/106	120	206/118
		Ether anesthesia			
15	186/98	75	196/96	130	198/110
20	190/100	80	188/96	135	204/108
25	196/104	85	190/102		
30	192/98	90	196/100		
Morphine sulphate subcutaneously— 0.01 gm. per kg.		95	198/108		
55	198/96				

cannulation and collection of the blood in a 50 cc. paraffined vessel, and then injected immediately into the recipient dog by means of a cannula in the external jugular vein. This method was necessarily slow, since only 50 cc. of blood could be transferred at one time. In the other method, which involved the use of heparin, the transfer was made in one operation. In this case the blood from the hypertensive dog was obtained from the cannulated renal vein. It is reasonable to suppose that, if the kidney is liberating a pressor substance, this material is present in greater quantities in renal vein blood than in blood drawn elsewhere. Also the kidneys of the recipient dog were tied off just previous to the experiment through lumbar incisions. After the blood had been collected and heparinized, it was immediately injected into the recipient dog through a cannula in the external jugular vein.

Six successful experiments have been performed, and in no case was there any evidence to show that the blood of the dogs with renal hypertension contained more pressor substance than normal blood. The blood pressures of the recipient dogs were not significantly altered by the transfusions of blood which they received from the hypertensive animals.

Summary. The transfusion of blood from dogs with hypertension caused by constriction of the renal arteries into normal dogs does not cause an elevation of blood pressure. In some cases relatively enormous amounts of blood were transferred—as much as 20% of the weight of the recipient. Even when this blood was taken from the renal vein and when the kidneys of the recipient were previously tied off, there was no elevation of the recipient's blood pressure. The method employed, then, gives no evidence for an increased amount of pressor substances in the blood of dogs with hypertension resulting from constriction of the renal arteries.

9044 P

Cytological Studies of Mammalian Embryonic Blood Cells.

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Blood cells are most readily classified when seen in blood smear preparations or dry imprints (smears) of tissues stained with Romanowsky dyes. In most laboratories, however, only paraffin sections are studied when the hematologist or pathologist is interested in the hemopoietic activity of spleen, liver, lymph nodes, etc.

American investigators have studied mammalian embryonic hemopoiesis from paraffin or celloidin sections only. It was deemed worthwhile to study embryonic hemopoietic organs using the dry imprint method, checking our observations with paraffin sections. The embryonic tissue (yolk-sac, liver, spleen, bone marrow) was touched gently to a chemically clean cover glass and waved vigorously until the imprinted material was dry. May-Grünwald-Giensa staining was used immediately as in the staining of blood smears. Preparations made in this way make possible a comparison between embryonic blood cells and elements seen in blood smears and organ imprints of the adult under normal or various physiologic and pathologic states. It is important to know which cells are found normally only in the embryo, and the elements of the embryonic hemopoietic organs which are identical with normal cells of the adult.

The material used for this study consisted of rat embryos from 9-22 days' gestation, plus various stages of rabbit, human, mouse and pig embryos and foetuses. The following were conclusions drawn:

1. The first circulating blood cells of the rat are not lymphocytes (Maximow, Jolly, Jordan), but are immature red cells of the embryonic megaloblastic series.

2. Two generations of red cells are produced in all mammalian embryos, the megaloblastic, formed primarily in the yolk-sac, and the normoblastic which appears in the liver in great numbers. The normoblasts of the liver are cytologically identical with the nucleated red cells of the fetal spleen and fetal and adult bone marrow. Megaloblasts are not the precursors of normoblasts. The normoblastic and megaloblastic series are distinct, with old and young stages in each line. Nuclear structure is the primary basis for distinguishing the megaloblast from the normoblast. In the immature stages dry

imprints are essential for classifying cells as either megaloblasts or normoblasts. In such preparations differences in the 2 series are constant.

3. The megaloblastic series of the embryo closely resembles the hemoglobiniferous series of pernicious anemia bone marrow during relapse. The cells of the rat yolk-sac are not, however, morphologically identical with the pathologic cells of pernicious anemia. The nuclear pattern of the embryonic megaloblast is coarser in the polychromatic stage. Since normoblasts, leukocytes and lymphocytes have the same nuclear patterns in all mammalian species, it is likely that this is true for the megaloblasts also. Thus, it might be assumed that in pernicious anemia red cell regeneration is similar to, but not identical with that of the embryonic yolk-sac.

4. The occasional presence of azurophilic granules in the cytoplasm of basophilic megaloblasts of the rat yolk-sac, an organ which produces only erythrocytes, proves that myeloid azure granulation in the cytoplasm of an immature blood cell does not necessarily mean the cell is a granulocyte precursor. Azurophilic granules have been observed by Jones[1] in pernicious anemia megaloblasts.

5. In the case of the rabbit the definitive (normoblastic) generation of red cells appears in the yolk-sac before hemopoiesis has begun in the liver. Only megaloblastic red cells are formed in the rat yolk-sac. Embryonic mammalian hepatic erythropoiesis is normoblastic only and primarily extravascular in location. The embryonic liver forms practically the same cellular elements as adult bone marrow, but the ratio of red cells to leukocytes is much higher in the liver.

6. Megakaryocytes of the embryonic liver are identical with those of the adult bone marrow, but it is questionable whether the yolk-sac forms megakaryocytes. As claimed by Storti[2, 3] hypertrophied megaloblasts with polymorphous nuclei and no azurophilic granules in the cytoplasm may represent the cells which have been interpreted as megakaryocytes by many authors. This point needs further study.

7. Megaloblasts circulate up to birth in animals with a short gestation period (rat, mouse). In the human and pig, where the gestation period is long, nucleated megaloblasts disappear from the circulation long before birth (in the human there are none after 12 weeks). Similar cells, according to many investigators, appear

[1] Jones, O. P., *Folia Hematol.*, 1936, **55**, 195.

[2] Storti, C., *Boll. d. Soc. Med.-Chir. Pavia*, 1932, **46**, 893.

[3] Storti, C., *Arch. Zool. Ital.*, 1935, **21**, 241.

again in the human adult only pathologically in the liver-principle deficiency anemias (pernicious anemia, tropical sprue, bothriocephalus anemia, pernicious anemia of pregnancy), the leukemias and agranulocytosis (one case in Dr. Hal Downey's collection).

9045

A Micro-Bioassay Method for Acetylcholine.*

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One of the most specific and sensitive tests for acetylcholine in body fluids is the leech muscle suspended in a saline bath containing eserine (Fuehner, Minz[1, 2]). When only small quantities of blood are available they must be diluted with sufficient saline to fill the bath. This procedure naturally decreases the sensitiveness of the test. Moreover, in smaller animals, the repeated drawing of several cc. of blood is often detrimental to the carrying out of a series of tests. The method presented here eliminates these disadvantages by suspending the leech in foam from small quantities of blood.

The leech is attached to a hook at the bottom of a glass bath (2x6 cm.), the other end leading to a writing lever. A narrow opening at the funnel-shaped bottom of the bath is connected with an air tank by means of rubber tubing, so that air is allowed to enter at the desired rate. A wire loop serves as a guide to prevent the blood soaked thread from adhering to the wall of the tube. The leech is prepared in the usual manner and is suspended in eserinized saline until it is relaxed. The saline is then drained completely and the blood which is to be tested is placed in the bottom of the tube. The air passing through the blood creates a foam, which passes over the muscle as it is carried upward. If there is acetylcholine present in the blood, the contraction of the muscle then starts immediately. (See Fig. 1.) In order to obtain uniform results, it is necessary to keep the amount of air entering the bath constant. After the foam has exerted its effect on the leech, the bath is washed with saline,

* Aided by a grant from the David Trautman Schwartz Research Fund.

1 Fuehner, H., *Biochem. Z.*, 1918, **92**, 347.

2 Minz, B., *Arch. f. exp. Path., u. Pharmakol.*, 1932, **167**, 85; **168**, 292.

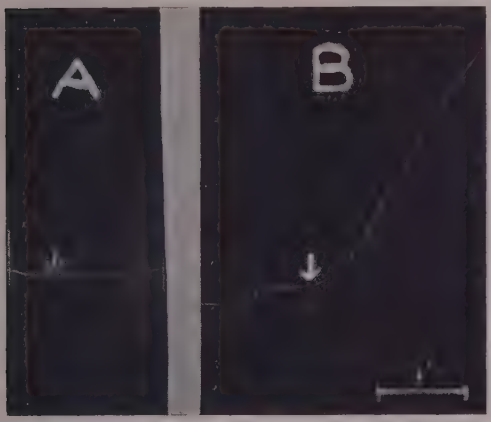

FIG. 1.
Leech Muscle, 1:7 Amplification.
A. Action of foam from 0.25 cc. blood from femoral vein of dog, containing Chlorazol Fast Pink and eserine 1:200,000.
B. Foam from 0.25 cc. blood from femoral vein of dog, containing Chlorazol Fast Pink, eserine 1:200,000 and acetylcholine chloride, 1:125 million.

with which it is left filled, until the muscle is relaxed and ready for another test. In order to prevent coagulation of blood and foam, a small quantity of an anti-coagulating substance must be added. This is done by rinsing the syringe with an 8% solution of "Chlorazol Fast Pink" before drawing the blood, the amount adhering to the syringe walls being sufficient to prevent coagulation. This solution may contain eserine in order to prevent the rapid destruction of acetylcholine. In experiments in which a known quantity of acetylcholine chloride (Hoffmann-LaRoche) was added to the eserinized blood, the resulting curve of the contraction of the leech caused by the blood foam could be matched by filling the bath with saline containing the same concentration of acetylcholine, provided the volume of the blood used was not less than 0.5 cc. If only 0.3 to 0.4 cc. of blood are available, their effects must be matched against foam from an equal quantity of old serum containing the known acetylcholine concentration. During a period of 2 or 3 minutes, foam from old serum (without acetylcholine) has no effect on the leech.

With this method we have been able to determine a given concentration of acetylcholine in as little as 0.3 cc. of blood or serum with a margin of error of about $\pm 20\%$. With less than 0.3 cc. of blood containing acetylcholine, the leech still contracts (See Fig. 1) but the margin of error becomes greater when the effect is matched with known concentrations.

We believe that the principle of this method can be successfully adapted to other bio-assays on isolated organs.

9046 P

Effect of "R" and "S" Forms of Chromogenic Acid-Fast Bacillus from Human Leprous Lesion on Rabbits.

J. R. Kriz. (Introduced by C. W. Duval.)

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The writer isolated[1] a "rough" and "smooth" form of a chromogenic acid-fast bacillus which was recovered from the human leprous lesion. The 2 forms differed both morphologically and culturally. A study was made to determine any difference in pathogenicity. Inoculations of rabbits began about a year ago. Large doses of the 2 forms were given at frequent intervals over a considerable length of time. Three rabbits were respectively injected with the "R" form subcutaneously, intraäbdominally and intranasally, and 3 other animals received the "S" form by the same routes. Preliminary to the injections of living bacilli, all 6 rabbits received several injections of a lepra broth-filtrate to produce, if possible, an allergic condition which would enhance the growth of the acid-fast bacilli *in vivo*.[2]

The "S" form produced the most striking results. The rabbits became markedly emaciated after approximately 7 months. Several subcutaneous nodules were noted at the sites of inoculations in the rabbits receiving the subcutaneous injections. Microscopic examination of ulcerated nodules revealed many acid-fast bacilli. The internal organs, especially the liver, spleen and kidneys, showed many large phagocytic cells, simulating lepra cells, containing acid-fast bacilli. Fibrosis and generalized thickening of the blood vessels was marked. Two rabbits showed paralysis of 2 or more extremities which probably was the result of extra- and intraneural fibrosis. Less conspicuous changes were noted in the rabbits injected with the "R" form. Emaciation was present but not to the degree seen in the rabbits that had received the "S" injections. Subcutaneous nodules developed but gradually disappeared without ulceration. Careful microscopic examination of the internal organs of the "R" animals revealed nothing characteristic of leprosy. No acid-fast bacilli were detected in any of the organs.

One of the objects of this investigation was to determine whether it is possible to induce in rabbits, by means of the chromogenic acid-

1 Kriz, J. R., Proc. Soc. Exp. Biol. and Med., 1936, **34**, 303.

2 Duval, C. W., and Gurd, F. B., *J. Exp. Med.*, 1911, **14**, 181.

fast bacillus isolated from the human leprous nodule, a disease comparable to human leprosy. The other object was to determine any difference in the pathogenicity of the 2 variants designated "R" and "S". The data indicate that the smooth form of this chromogenic acid-fast bacillus is the more pathogenic for rabbits.

9047 P

Action of Acetylcholine on Heart and Skeletal Muscle.*

M. BODANSKY AND PAUL BRINDLEY. (With the technical assistance of C. L. Herrmann and Katherine R. Campbell.)

From the Laboratories of the John Sealy Hospital and the Departments of Pathology and Pathological Chemistry, University of Texas Medical School, Galveston.

Hall, Ettinger and Banting[1] found that long-continued administration of acetylcholine produced myocardial and coronary artery damage, the effects being more severe and extensive in old than in young animals. In pursuing our interests in the problem of myocardial and skeletal muscle disease in relation to the creatine reserve, we attempted to study the effects in the rat. As in the experiments reported from the Banting Institute, acetylcholine bromide (Eastman-Kodak) and acetylcholine iodide (Hoffmann-La Roche) were used. The dose was 10 mg. of acetylcholine daily, administered in a single dose or in 2 divided doses. The rats were 5-6 months old at the beginning of the experiments and the average weight exceeded 300 gm. Fourteen of the 17 rats on which the present report is based received the drug for a period of 85-90 days, at which time they were sacrificed.

Within a few seconds after each injection, the characteristic effects of the drug referable to autonomic activity, *i. e.,* profuse salivation and lacrimation, accelerated respiration and heart rate, were manifested. These symptoms gradually subsided during the succeeding 10 minutes.

Heart. The changes in the heart were somewhat variable, but in most animals there was present some degree of myocardial degeneration, hyaline change and beginning fibrosis. A striking feature

* Aided by a grant from the National Research Council.

1 Hall, G. E., Ettinger, G. H., and Banting, F. G., *Canad. Med. Assn. J.*, 1936, **34**, 9.

was the patchy distribution of the small areas of degeneration, often verging on necrosis. Infiltration of these areas with inflammatory cells, vacuolization, hydropic change, thickening of the vessel walls due mainly to endothelial proliferation, and scattered small hemorrhages into the myocardium were likewise in evidence. Despite these changes it was remarkable to discover that the creatine concentration was essentially unaffected (159-237 mg., average 206 mg.) in all the animals that survived the period of experimentation (15 out of 17). In one rat which died in heart failure, the auricles were greatly dilated and there was present generalized edema with ascites. The creatine concentration of the myocardium was only 106 mg. In this animal there was thickening of the walls of the blood vessels due to fibrous tissue production. A nerve ganglion on the wall of the heart showed marked fibrosis. A low value for creatine (113 mg.) was observed in a second animal in which death was associated with considerable accumulation of bloody fluid in the pericardial sac. It, therefore, appears that although the creatine reserve of the myocardium was lowered in the 2 cases just cited, nevertheless severe myocardial damage existed in the greater proportion of animals without any significant change in creatine concentration. This observation confirms our experience in clinical heart disease as disclosed by the results of an extensive series of analyses of human hearts (Bodansky, Pilcher and Duff, unpublished data).

Skeletal Muscle. Rarely, focal areas of myositis with degeneration were noted and somewhat more frequently hyaline change and fibrosis were present, but for the most part the muscles appeared essentially normal, except for hyperplasia of the endothelium of the blood vessels which was a relatively constant finding. Without exception the creatine concentration remained essentially within normal limits.

Muscle Metabolism. A proportion of the rats were kept in suitable metabolism cages for periods of 30 days, or longer, and the urine, collected at intervals of 48 hours, was analyzed for nitrogen, creatine, creatinine, and guanidoacetic acid. Considering the physiological properties of acetylcholine in the dosage administered, it was of interest to find that there was no significant change from the normal in the excretion of these constituents.

9048

Hormone Production in the Undescended Testis.

F. M. Hanes and Chas. W. Hooker. (Introduced by Harold Cummins.)

From the Department of Medicine, School of Medicine, Duke University.

The level of production of the "male hormone" in cryptorchid as compared with scrotal testes is of interest on two counts. Failure of the testis to descend is a fairly common defect in development, and an altered hormone-producing capacity of the retained testis may have a bearing on the origin and perhaps even on means of correcting the defect. Moreover, the inhibited differentiation of the seminiferous tubules in the cryptorchid testis, accompanied by evident increase of the interstitial elements, renders it interesting in relation to the question of the site of production of the testis hormone.

Apparently there have been few studies concerned with hormone production in the undescended testis. Early workers merely noted that the secondary sex characters are essentially normal in cryptorchid animals (Bouin and Ancel[1]). The first quantitative study seems to be that of Moore and Gallagher[2] who found, in testing the cryptorchidized guinea pig by the electric ejaculation method, that hormone production was as great as normal. Jeffries,[3] utilizing the cytological signs in seminal vesicle and prostate, observed no castration changes in rats 60 days after cryptorchidizing, from which it was thought that there was no diminution in hormone production. Nelson,[4] using the same tests, found in rats 240 days or longer after being cryptorchidized normal prostates, but seminal vesicles showing castration changes, an observation indicating reduced hormone production in the operated animals. It is to be noted that these authors used as criteria of hormone production not quantity of hormone itself but hormonally regulated characters in their cryptorchid animals.

The present report is based on a study of 2 lots of mixed cryptorchid and normal testes of swine obtained in New York City through the assistance of Dr. C. A. Slanetz. The testes were placed on CO_2-ice upon being taken from the animals and were packed in CO_2-ice for shipping. When received at the laboratory the tissue

1 Bouin, P., and Ancel, P., *Arch. de Zool. Exp. et Gen.*, 4th ser., 1903, **1**, 437.
2 Moore, C. R., and Gallagher, T. F., *Am. J. Anat.*, 1930, **45**, 39.
3 Jeffries, M. E., *Anat. Rec.*, 1931, **48**, 131.
4 Nelson, W. O., *Anat. Rec.*, 1934, **58**, 30 (suppl.).

was still frozen solid. A fragment of each testis was submitted to frozen section and stained with polychrome methylene blue in order to differentiate the normal from the cryptorchid gonads. The tissue was extracted by the method of Gallagher and Koch[5] as far as the cold acetone stage. The cold acetone-soluble material was dissolved in olive oil and assayed upon White Leghorn capons by the method of the same authors.[6]

The first lot of testes yielded 232.5 gm. of normal testis and 694.5 gm. cryptorchid. The normal tissue was found to contain 1 bird unit in each 38.7 gm., while the cryptorchid tissue contained 1 bird unit in 86.7 gm. of tissue. The second group contained 134 gm. normal testis and 964 gm. cryptorchid testis. Here the scrotal testes contained 1 bird unit in 27.0 gm. and the cryptorchid tissue 1 bird unit in 53.5 gm. of tissue. These weights are based on testis tissue stripped of all its coverings.

The values found indicate that in the pig the cryptorchid testis contains approximately one-half as much hormone per unit weight as does the scrotal testis. And it may be emphasized that here we have determined the quantity of hormone itself and not the relative masculinity of the animals, in which the hormone may be only one regulating factor. The explanation of the difference in potencies of the 2 lots of testes, especially marked in the cryptorchids, has not been explained; but it will be noted that the values obtained by us for the normal tissue agree quite closely with the potency of bull testis tissue as reported by Womack and Koch.[7]

The bearing of the present findings on the question of the site of production of the testis hormone is somewhat uncertain. It is clear that hormone production is continuing notwithstanding the state of the tubular tissue as described by Hanes[8]; however, despite probable hypertrophy of the interstitial cells, the rate of production is distinctly less than normal. In this connection the finding of Moore and Samuels[9] that the testis may present an essentially normal structure and yet secrete no hormone is of interest.

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5 Gallagher, T. F., and Koch, F. C., *J. Biol. Chem.*, 1929, **84**, 495.

6 Gallagher, T. F., and Koch, F. C., *J. Pharm. Exp. Therap.*, 1930, **40**, 327.

7 Womack, E. B., and Koch, F. C., *Endocrinology*, 1932, **16**, 267.

8 Hanes, F. M., *J. Exp. Med.*, 1911, **13**, 338.

9 Moore, C. R., and Samuels, L. T., *Am. J. Physiol.*, 1931, **96**, 278.

9049

Determination of Small Amounts of Morphine in Blood.

James W. Mull.

From the Laboratory of the Maternity Hospital, Cleveland.

The fate of morphine in the animal body is a question that has stimulated much excellent work and the development of many methods for the determination of the drug. We do not have space to enumerate, much less to discuss, these methods, 2 of the more recent examples of which are those described by Wolff, Riegel, and Fry[1] and by Abe and Uchida.[2] Most of these methods were unsuited to our purpose since they were not designed for use with blood, and the others required more material or a more elaborate procedure than we wished to employ.

The method herein described has the advantage of simplicity, takes but a few hours to complete, and gives quantitative results with small amounts of blood. It is based on that of Sanchez,[3] which was designed for the determination of morphine in pure solution. We have modified it for use with blood filtrates, deproteinized by the method of Somogyi,[4] introducing scopolamine to bring down the precipitate and permit the removal of the reacting solutions. The method in detail is as follows: A 1-to-10 filtrate is prepared by laking 1 part of blood with 7 parts of water. To this is added with constant stirring 1 part 10% zinc sulphate and 1 part 0.5 N sodium hydroxide.* After standing 10 minutes the precipitate is centrifuged down and the supernatant liquid filtered through No. 2 Whatman paper. Treated in this manner 5 cc. blood yield over 30 cc. filtrate.

To 20 cc. of the filtrate, equivalent to 2 cc. blood, is added 2.5 mg. of scopolamine hydrobromide, from a solution containing 1 mg. per 1 cc. (Proportionally smaller amounts may be used if the concentration of morphine is high.) The morphine is then precipitated by 0.4 cc. of Wavelet's solution.† The material is thoroughly mixed

1 Wolff, W. A., Riegel, C., and Fry, E. G., *J. Pharm. and Exp. Therap.*, 1928, **33**, 329.

2 Abe, K., and Uchida, T., *Jap. J. Med. Sci., Pharm.*, 1934, **8**, 89*.

3 Sanchez, Juan A., *La Semana Medica*, 1930, ii, 333.

4 Somogyi, M., *J. Biol. Chem.*, 1930, **86**, 655.

* The $ZnSO_4$ and the NaOH must be so related that 10 cc. of the former require from 10.8 to 11.2 cc. of the latter to produce a permanent pink color with phenolphthalein.

† Wavelet's solution is made by dissolving 7 gm. sodium carbonate and 1 gm.

by stirring or shaking, and set in a cold place for about an hour. The precipitate is then centrifuged down. The clear liquid is decanted and discarded. After draining and wiping out the tubes to remove all traces of fluid, the precipitate is suspended in 1.5 cc. water and dissolved in 10 drops of a 2% solution of ammonium hydroxide. Solution is promoted by stirring and shaking and is accompanied by the development of a blue color, which persists for at least 20 minutes. This color is compared with that of standards either in conical centrifuge tubes or in a microcolorimeter.

Standards are prepared from blood. Pooled specimens are more easily obtained, are entirely satisfactory, and may give a slightly more uniform standard. The desired amounts of morphine are added to the whole blood before laking. The specimens are then treated exactly like the unknowns, using the same volumes. These protein-free filtrates keep well with refrigeration, and may be used for 2 or 3 weeks, after which there seems to be some loss of strength, as shown by weaker color development. It has been our custom to precipitate the proteins a few minutes after the specimens were drawn. In one case, however, the whole blood was permitted to stand 3 days, with only a slight loss of morphine resulting.

Standards, without morphine, prepared by this method are colorless. With 0.01 mg. of morphine a clear blue color is obtained. Although not directly proportional, the color varies with the concentration of the morphine, 0.005 mg. giving a distinct color, and 0.002 mg. standards being definitely distinguishable from the blanks. It is advisable to prepare a series of standards varying by 0.005 mg. to cover the expected range. Unusual retention of uric acid, over 25 mg. per 100 cc., interferes with the determination, giving a faint blue color with the reagents.

The scopolamine promotes the precipitation of the morphine. It produces no color, since the blanks are water clear; but without it, smaller amounts of morphine are lost. It is apparently most effective in the proportion of 0.7 mg. per 5 cc. of filtrate. If too much is used it does not dissolve readily in the ammonium hydroxide, and tends to color the final solution green. The limit is about 2.5 mg., the amount necessary for 20 cc. of filtrate.

Stronger solutions of ammonium hydroxide, or larger amounts, dissolve the precipitate more rapidly, but fade the blue color. The proportion of 0.1 cc. of the precipitating reagent to 5 cc. of filtrate

disodium acid phosphate in 25 cc. water. To this is added 3.5 gm. freshly calcined molybdic acid, and, following complete solution, 10 cc. concentrated nitric acid. The solution is allowed to stand over night, then filtered. It keeps indefinitely at room temperature.

seems entirely adequate. Larger amounts tend to be retained in the residue and to interfere with the final color. In preparing the standards the morphine must be added to the whole blood, since additions to the filtrate give darker colors. This confirms earlier work[5] that there is some loss of morphine with the proteins, but there is no reason to believe that the loss is not as great with the standards as with the unknowns. This loss is greater with a more concentrated filtrate, as a 1 to 5 dilution, and less with a 1 to 20. The latter is unsatisfactory, however, since it increases the volume of the filtrate, and the determinations as now run are limited to 20 cc.

We have used this method successfully with rabbits, and have found the morphine concentration of the blood to vary with both dosage and time. A 6 kg. rabbit given 64.8 mg. (1 grain) morphine intramuscularly showed 0.014 mg. per 1 cc. blood after 35 min., and the same concentration at the end of 1 hour. Two others, weighing 3.7 and 3.4 kg., were given 16 mg. each. After 45 min. the first had a concentration of 0.004 mg. per 1 cc., and after 1½ hours 0.007 mg. The 2nd had 0.006 mg. after 35 min. These showed a satisfactory uniformity on the basis of the dosage per kilo of body weight. A fourth rabbit, weighing 3.6 kg. and given 16 mg., showed a lower concentration, 0.0035 mg. after 1 hr., and 0.0055 mg. after both 2 and 3¾ hours. The method is, therefore, satisfactory for animal experimentation.

It has not proven sufficiently delicate, as it now stands, for use with humans. The blood following the therapeutic dosage of 10 to 16 mg. does not give any color. That from 2 patients, the first given 3 injections of 16 mg. morphine and 1 of 10 mg., at 3 hr. intervals, and the second a single injection of 32 mg., did show a faint color, using 20 cc. of filtrate, but not sufficient for a quantitative estimation. These results indicate, however, that the blood of addicts would contain sufficient morphine to permit accurate determinations.

We have confirmed the findings of Teruuchi and Kai[5] that morphine is divided between cells and plasma. This necessitates using whole blood for all morphine determinations.

Summary. A simple, inexpensive colorimetric method, adapted from that of Sanchez, is herein described by which morphine may be determined in blood when the concentration is not less than 0.0025 mg. per 1 cc. The method is practical for animal experimentation, but is not sufficiently delicate, as given, for use with human subjects receiving therapeutic doses of morphine. Whole blood is used since morphine can be found in both cells and plasma.

[5] Teruuchi, Y., and Kai, S., *J. Pharm. and Exp. Therap.*, 1927, **31**, 177.

9050 P

Creatine Content of Human Voluntary Muscle.

J. F. Corsaro. (Introduced by V. C. Myers.)

From the Department of Biochemistry, School of Medicine, Western Reserve University, Cleveland.

Although considerable data have been published on the creatine content of the muscle of laboratory animals, data on the creatine content of human voluntary muscle are somewhat meager. For this reason a study was made on material obtained from 74 human autopsy cases (less than 36 hours post mortem). Creatine was determined on 3 voluntary muscles, namely the psoas major, rectus abdominis and sternocleidomastoid, the average for the respective muscles being 402, 405, and 388 mg. per 100 gm. of fresh muscle.

If the average data secured on muscle obtained at autopsy may be taken as representing the normal creatine content of human voluntary muscle, the concentration is in the neighborhood of 400 mg. of creatine per 100 gm. of muscle. Although in this series there was a fairly even balance between groups of cases showing high and low creatine values, it is possible that the average value for strictly normal individuals is somewhat in excess of 400 mg.

It would appear on the basis of the data obtained on the 3 muscles studied, and also on unpublished data from this laboratory by Linegar and by Mangun for the pectoralis major, that the creatine concentration of various striated muscles in the human is essentially the same. The findings for these muscles may differ somewhat in an individual but the averages for a group are of the same order of magnitude.

The results of this work indicate that relatively high values for muscle creatine, to about 550 mg., may be found in uremia, pneumonia, tuberculosis, early malignancy, and in some cases with circulatory involvement, while low values (as low as 250 mg.) may be encountered in late malignancy, acute inflammatory diseases, uremia plus heart failure, and in some cases with circulatory involvement.

9051 P

Electrolyte Content of Human Autopsy Tissue.*

EDWARD MUNTWYLER, RAMON F. HANZAL, GEORGE H. MANGUN AND CHARLES T. WAY.

From the Department of Biochemistry and the Institute of Pathology, School of Medicine, Western Reserve University, Cleveland.

During the last few years increasing attention has been given to the electrolyte content of tissues (particularly heart tissue). Some time ago we began a study with the purpose of comparing the electrolyte content of tissues from cases with renal disease with that of tissues from patients who had died with other diseases. At present our series does not include a sufficient number of renal cases to make this comparison significant. That is, since the electrolyte content of a given tissue may vary quite widely, a fairly large number of cases must be obtained. Therefore, the purpose of the present report is to present a summary of the results which have been obtained on a series of miscellaneous cases.

The tissues (right ventricle, left ventricle, skeletal muscle, kidney, liver and spleen) were analyzed for water, chloride, phosphorus, sodium, potassium, calcium and magnesium in a manner similar to that outlined by Cullen and Wilkins.[1] Table I presents the maximum, minimum and average values found. The parentheses indicate the number of cases used in computing the average. It will be seen that in addition to the analyses made by Cullen, Wilkins, and Harrison[2] and Wilkins and Cullen,[3] the present study includes the determination of the electrolyte concentration of spleen, the determination of the calcium and magnesium concentrations of kidney and liver and the determination of the sodium concentration of muscle, kidney and liver. The difference between the electrolyte content of right ventricle and left ventricle has already been discussed by the above workers. The data of the present series are in agreement with their findings with the exception that the average magnesium content of the right ventricle is slightly greater than that of the left ventricle. The above workers found that the left ventricle had a slightly greater content of magnesium than the right ventricle. In general, it will be seen from the average values obtained on the 6

* Aided by a grant from the Josiah Macy, Jr., Foundation.

1 Cullen, G. E., and Wilkins, W. E., *J. Biol. Chem.*, 1933, **102**, 403.

2 Cullen, G. E., Wilkins, W. E., and Harrison, T. R., *J. Biol. Chem.*, 1933, **102**, 415.

3 Wilkins, W. E., and Cullen, G. E., *J. Clin. Invest.*, 1933, **12**, 1063.

TABLE I.
The Electrolyte Content of Human Autopsy Tissue.
(Values expressed in terms of mg. per 100 gm. of fresh tissue)

		Right Ventricle		Left Ventricle		Skeletal Muscle		Kidney		Liver		Spleen	
Water	Max.		84.7		80.4		83.3		85.1		80.4		81.2
	Min.		73.8		75.8		71.6		79.6		68.8		74.4
	Aver.	(10)	79.9	(11)	79.0	(15)	77.9	(14)	82.4	(15)	76.1	(7)	78.3
Chloride	Max.		222		195		167		276		207		209
	Min.		136		98		55		155		72		171
	Aver.	(10)	182	(11)	139	(15)	96	(14)	208	(15)	149	(7)	192
Phosphorus	Max.		172		210		252		232		291		236
	Min.		126		132		92		113		189		142
	Aver.	(11)	147	(18)	180	(15)	169	(14)	151	(15)	226	(7)	201
Sodium	Max.		197		132		232		240		233		140
	Min.		75		83		52		91		88		113
	Aver.	(8)	126	(11)	99	(15)	102	(14)	143	(15)	114	(7)	127
Potassium	Max.		240		322		397		216		304		312
	Min.		146		184		251		114		138		197
	Aver.	(11)	206	(18)	269	(15)	324	(14)	175	(14)	233	(5)	250
Calcium	Max.		15.1		10.8		9.2		12.5		8.9		8.9
	Min.		4.4		4.7		2.6		3.8		2.0		2.6
	Aver.	(8)	8.4	(10)	7.2	(15)	6.5	(14)	7.1	(14)	6.5	(6)	5.4
Magnesium	Max.		28.3		26.1		29.8		19.3		24.2		18.7
	Min.		13.4		10.4		12.7		12.6		10.6		12.3
	Aver.	(8)	20.3	(10)	18.7	(15)	17.0	(14)	16.1	(14)	16.3	(6)	15.0

tissues examined that the water concentration is highest in the kidney and lowest in the liver; the chloride is highest in the kidney and lowest in skeletal muscle; the phosphorus is highest in the liver and lowest in the right ventricle; the sodium is highest in the kidney and lowest in the left ventricle; the potassium is highest in skeletal muscle and lowest in kidney; and calcium and magnesium are highest in the right ventricle and lowest in the spleen.

9052 P

Ascorbic Acid Content of Tissues Following Ether Anesthesia.

DONALD E. BOWMAN AND EDWARD MUNTWYLER.

From the Department of Biochemistry, School of Medicine, Western Reserve University, Cleveland.

It was recently reported by us[1] that following ether anesthesia in dogs, rats and guinea pigs the urinary excretion of ascorbic acid is increased. This excess excretion, which amounts to a 10 to 15 fold increase in the case of the dog but which is somewhat less in the case of rats and guinea pigs, is only of short duration. That is, the excretion falls to the normal level or slightly below in the second 24-hour urine collection following the anesthesia.

It was naturally of interest to determine whether similar periods of ether anesthesia as employed in the above studies are followed by changes of the ascorbic acid content of the tissues. Therefore, rats and guinea pigs, after being maintained on a constant diet for some time, were subjected to 2-hour periods of ether anesthesia.

TABLE I.

The Ascorbic Acid Content of Rat and Guinea Pig Tissues Following Ether Anesthesia.

No. of Animals		Tissues					Remarks
		Muscle	Kidney	Liver	Adrenal	Spleen	
		Ascorbic acid, mg. per 100 gm. fresh tissue.					
15	Aver.	6.2	14.4	26.6	337		Normal adult rats.
	Max.	8.5	19.8	35.3	447		
	Min.	4.2	10.5	16.2	152		
8	Aver.	7.6	27.2	45.1	287		Rats killed immediately after 2 hr. ether anesthesia.
	Max.	8.8	38.2	57.8	353		
	Min.	6.7	19.1	34.1	225		
6	Aver.	6.8	21.9	40.9	248		Rats killed 4 hr. after the end of 2 hr. anesthesia.
	Max.	8.9	32.6	52.8	335		
	Min.	4.9	12.7	34.6	126		
5	Aver.	6.3	18.7	32.1	184.6	58.1	Normal adult guinea pigs.
	Max.	7.5	20.3	39.4	215	61.7	
	Min.	5.8	17.6	26.7	168	53.8	
5	Aver.	7.0	17.6	33.9	116.9	53.9	Guinea pigs killed immediately after 2 hr. anesthesia.
	Max.	8.0	20.1	42.0	141.9	58.3	
	Min.	6.0	13.5	22.4	91.2	48.1	
5	Aver.	6.0	14.6	21.6	113.8	44.5	Guinea pigs killed 2 hr. after the end of 2 hr. anesthesia.
	Max.	6.2	17.6	23.8	150	50.5	
	Min.	5.8	11.4	19.3	86	40.5	

[1] Bowman, D. E., and Muntwyler, E., *J. Biol. Chem.*, 1936, **114**, XIV.

The animals were killed by a blow on the head either immediately after the anesthesia or 2 to 4 hours later. The ascorbic acid content of the various tissues was determined by titration with 2,6 dichlorophenol indophenol according to the procedure outlined by Birch, Harris and Ray.[2]

Table I presents the results obtained. In the case of rats which were killed immediately following the anesthesia, the average ascorbic acid content of kidney and liver is definitely higher than that found in the controls. On the other hand, the average ascorbic acid content of the adrenals is reduced. When the rats were killed 4 hours following the anesthesia, the ascorbic acid content of kidneys and liver, though less than that observed in the animals killed immediately following the anesthesia, is still definitely above the control level. At the same time the ascorbic acid content of the adrenals shows a continued decrease. Somewhat different changes were observed in the case of guinea pigs. There appears in this case to be a definite tendency to a reduced ascorbic acid content of kidneys, liver, adrenal and spleen tissue. That is, the ascorbic acid content of these 4 tissues in animals killed 2 hours following ether anesthesia is definitely below the control level.

Experiments are being continued with the object of finding an explanation for these results.

9053

Relationship Between Brain Potentials and Some Other Physiological Variables.

DONALD B. LINDSLEY AND BORIS B. RUBENSTEIN.* (Introduced by T. Wingate Todd.)

From the Brush Foundation, Western Reserve University.

Most prominent in the records of the electrical activity from the brain in intact human subjects are large and fairly rhythmic oscillations of potential called alpha waves. In a recent study of brain potentials in children and adults by Lindsley[1] the average frequency of the alpha rhythm in 54 adults was found to be 10.4 per second with a range of variation from 8 to 12 per second. Under normal

2 Birch, T. W., Harris, L. J., and Ray, S. N., *Biochem. J.*, 1933, **27**, 590.

* General Education Board Fellow.

1 Lindsley, D. B., *Science*, 1936, **84**, 354.

conditions the frequency for any one individual was remarkably constant, often varying by less than 1 cycle per second over a period of months and only occasionally by as much as 2 cycles per second. In children the rhythmic alpha waves first appeared at about 3 months of age and at a frequency of 3 to 4 per second. The frequency increased with age until the adult average was reached at 8 to 10 years of age. A slight rise in frequency above the adult level occurred between 10 to 12 years of age.

The present study is concerned mainly with the frequency variations observed in adults, since the various processes of growth and development make difficult certain comparisons in children. An attempt has been made to determine the relationship between the frequency of the alpha waves and some other physiological variables such as metabolic rate, heart rate, blood pressure, rectal temperature and respiration.

Thirteen adults, 12 women and 1 man, were used as subjects. Of these, 4 women medical students ranging in age from 21 to 31 years were studied every morning for 32 or 34 consecutive days. All records were obtained early in the morning under basal conditions. Rectal temperatures were taken by the women on awakening and before getting out of bed. On arrival at the laboratory one-half hour rest was required before records were obtained.

Brain potentials were recorded from the surface of the scalp by means of electrodes attached just posterior to the parieto-occipital fossa, about 2 inches to the right of the mid-line. The records were obtained with the subject lying on a cot in a dark room. Appropriate amplifier-oscillograph systems were employed in the recording of the brain potentials and electro-cardiograms. Repeated readings of systolic and diastolic blood pressure were made by the auscultatory method. Two 8-minute records of basal metabolism (expressed in total calories per hour) were obtained with the Benedict-Roth apparatus.

Figure 1 shows typical records of brain potentials, heart rate (electrocardiogram Lead II) and respiration taken simultaneously. Such records were obtained from the 4 subjects during a control period prior to the first basal, just before the end of the first basal after breathing oxygen for 8 minutes, during a second control period following the second basal, and finally at the end of a period during which the oxygen in a mixture (90% nitrogen, 10% oxygen) was exhausted and a condition of anoxia approached. Records were taken only during a control period for 9 other subjects who were studied but once.

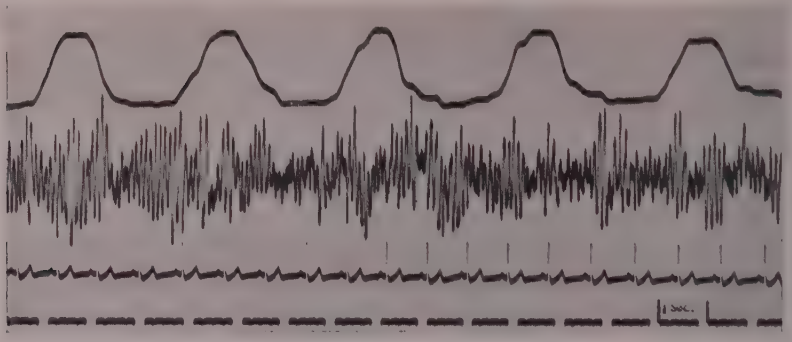

FIG. 1.

Typical record of respiration, brain potentials and heart rate (electrocardiogram Lead II).

Analysis of the records in terms of frequency and amplitude of the alpha waves revealed no significant differences between the first control period and the breathing of oxygen, nor between the second control period and the period of exhaustion of oxygen. Gibbs, Davis and Lennox[2] reported an increase in amplitude and a decrease in frequency of the waves on breathing pure nitrogen, and especially marked changes when a state of confusion or unconsciousness were produced. Our failure to obtain similar changes may have been due to the fact that the experiment was never continued beyond an initial state of discomfort so that a prolonged period of complete anoxia was probably never attained.

Using the data from the first control period correlation coefficients were obtained between the frequency of the alpha waves and each of the other daily measures of physiological activity. The correlation coefficients were inconsistent from subject to subject and in general were low and without prediction value. This may have been due to the fact that the range of variation of the frequency of the alpha waves for any one individual was small, and also that the variability of certain of the measures in these 4 subjects was high during a period of from 1 to 2 weeks near the middle of the menstrual cycle,[3] which may have tended to obscure relationships between measures for any one subject. In addition, correlation ratios showed some of the regressions to be non-linear, thus making the correlation coefficients in these particular cases invalid.

The variation in the frequency of the alpha waves from subject

[2] Gibbs, F. A., Davis, H., and Lennox, W. G., *Arch. Neurol. and Psychiat.*, 1935, **34**, 1133.

[3] Rubenstein, B. B., and Lindsley, D. B., Unpublished data.

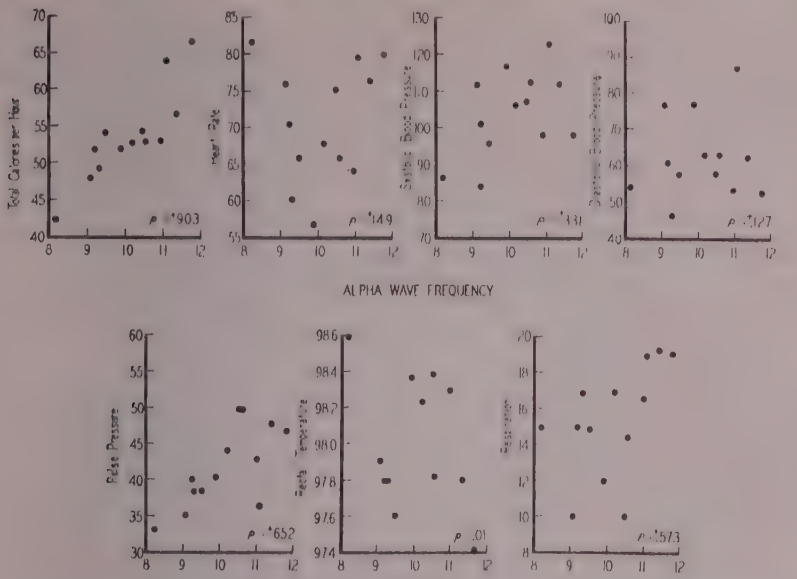

FIG. 2.

Graphical representation of data from Table I and the correlation coefficients between the frequency of alpha waves and the other physiological variables for 13 subjects.

to subject was much greater than that for any one individual and it is only when comparisons of the measures from one subject to another are made that significant relationships are observed. Table I shows the means and standard deviations of all the measures for the 4 subjects studied 32 or 34 days and also the daily values for each of the other 9 subjects. Figure 2 shows these data plotted against the frequency of the alpha waves for each of the 13 subjects. Rank difference correlation coefficients are also given. The relationship between the frequency of the alpha waves and metabolic rate (total calories per hour) is very significant. Pulse pressure and respiration show less significant relationships to the frequency of the alpha waves, but nevertheless values which approach significance. There was not a significant relationship between the frequency of alpha waves and the other measures (systolic and diastolic blood pressure, heart rate, and rectal temperature).

The correlation between alpha wave frequency and basal metabolic rate in *calories per square meter per hour* is of doubtful significance ($\rho = +.396$). The relationship between frequency and surface area or body size is significant ($\rho = +.715$); that between total calories per hour and surface area is $\rho = +.719$.

TABLE I.

Means and Standard Deviations of Measures for 4 Subjects Studied 32 or More Consecutive Days and Single Day Values for 9 Other Subjects.

Subject	Age	Alpha wave freq.	Total cal./hour	Heart rate	Syst. blood press.	Diast. blood press.	Pulse press.	Rectal Temp.	Respiration
A	30	10.58	52.75	66.16	113.38	63.31	49.72	97.82	14.44
		.45	3.89	3.87	3.21	4.71	4.09	.36	2.49
B	32	10.23	52.80	68.06	107.09	63.09	44.00	98.23	16.97
		.40	2.71	4.55	3.09	3.54	3.73	.38	1.78
C	23	11.39	56.73	77.47	111.59	63.16	48.44	97.81	19.19
		.47	4.51	4.76	4.63	5.67	4.53	.28	2.08
D	22	11.00	53.41	64.18	97.74	54.24	43.21	98.13	16.71
		.40	3.25	5.26	3.17	3.22	2.74	.33	2.55
E	27	8.2	48.1	82.	87.	54.	33.	98.6	15.
F	39	9.9	51.9	56.	118.	78.	40.	98.4	12.
G	30	10.5	54.3	76.	108.	58.	50.	98.4	12.
H	30	9.2	51.7	72.	102.	62.	40.	97.8	15.
I	22	9.5	53.6	66.	96.	58.	38.	97.6	15.
J	23	11.77	66.8	81.	99.	52.	47.	97.4	19.
K	36	9.1	47.8	77.	113.	78.	35.	97.9	10.
L	24	9.3	49.1	60.	84.	46.	38.	97.8	17.
M*	36	11.05	64.0	80.	124.	88.	36.	.....	19.

*Male subject.

Further suggestion of the relationship between the frequency of the alpha waves and metabolic rate was found in an individual subject whose metabolic rate was increased by taking thyroxin. Control records showed the frequency of the alpha waves to be 10.5 per second and the total calories per hour to be 53.7. Three days after 1.6 mg. of thyroxin had been administered, when the metabolic rate had reached its highest value (59.0 calories per hour), the frequency of the alpha waves had increased to 11.4 per second. On the fourth day the total calories per hour had dropped to 56.7 and the frequency to 10.6 per second.

Although the basic rhythm of the alpha waves is undoubtedly established in adults by other factors, it appears from our results that metabolic rate largely determines the differences in frequency (8 to 12 per second) observed in adults. It is possible also that metabolic rate has much to do with variations in the frequency observed in children.

Summary. A very significant positive relationship ($\rho = +.903$) between the frequency of brain potentials (alpha waves) and metabolic rate (total calories per hour) has been demonstrated in 13 adult subjects. A similar relationship was demonstrated in a subject whose metabolic rate was elevated by 1.6 mg. of thyroxin. The relationships between the frequency of alpha waves and pulse pressure and respiration barely approached significance, whereas those between the frequency of the alpha rhythm and systolic and

diastolic blood pressure, heart rate and rectal temperature were not significant. No variation in the frequency or amplitude of the alpha waves was observed in 4 subjects during the breathing of oxygen or during the breathing of a mixture (90% nitrogen, 10% oxygen) until the oxygen had been exhausted.

9054

Unrecorded Form of *Bacterium aurescens*, Sole Colon-Group Representative in a Fecal Specimen.

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Chromogenic members of the colon-group are not unknown. MacConkey[1] listed yellow colon-group liquefiers from horse feces, pond water, rain water, roof washings, oats, beans, malt and ears of corn, and he reported a yellow *B. coli communis* from rain water. Rogers, Clark, and Lubs[2] reported that but few grain cultures are without pigment, many being decidedly chromogenic. They stated that this property is correlated with other characters and consequently is of value in classification. In a collection of colon-bacteria from human feces they found chromogenesis almost entirely absent. All their fecal cultures gave a faint yellow color but this was so slight and showed so little variation that it was of no value in differentiation. They did state, however, that there were a few exceptions to this rule. Wood[3] reported that 7 of 20 colon-group strains isolated from grains, hay and dried eggs and milk produced yellow pigment. In his Pocomoke river series Perry[4] encountered 5 cultures of aërobic, non-sporulating bacteria producing gas from lactose which produced a distinct yellow pigment. He excluded these chromogenic strains from consideration as fecal *coli*.

On January 14, 1936, a fecal specimen was received for study. Although the patient complained of certain general symptoms none referred to the gastro-intestinal tract and the analysis was under-

1 MacConkey, *J. Hygiene*, 1909, **9**, 86.

2 Rogers, Clark, and Lubs, *J. Bact.*, 1918, **3**, 231.

3 Wood, *J. Hygiene*, 1919, **18**, 46.

4 Perry, *Am. J. Hygiene*, 1929, **10**, 580.

taken as part of a routine rather than as an indicated procedure. A suitable saline suspension was at once prepared and adequately plated on citrated agar, blood agar, and Endo's agar. A small bit of the stool was placed in a large tube containing 30 cc. of lactose-indicator-broth. In this enriched medium typical acid- and gas-formation was observed in 24 hours. No colonies appeared on the citrated agar although it was heavily inoculated. On the 3 blood agar plates all colonies were inactive and of a size and type suggestive of colon bacilli. On the 3 Endo's plates all colonies were "typical *coli*," showing the well known metallic sheen; and were flat, round, regular and smooth. Twenty-nine colonies were picked from the blood-agar and Endo's plates, 4 for repeated purification and detailed pure-culture study and 25 for a check on their ability to utilize citrate as a sole source of carbon. None of the 29 colonies developed on citrate.

As the pure-culture study proceeded it was noticed that a colored sediment formed in such uncolored broth mediums as those used for the indol, methyl-red, and Voges-Proskauer tests. When grown on ordinary nutrient agar the strains produced a distinct golden-brown insoluble pigment. This pigment formed as well at 37°C. as at room temperature and was as distinct and of practically the same color as that observed for a typical *Staphylococcus aureus*. That pigment-formation is a true biological function of these strains was shown by the constant appearance of pigment on all mediums capable of revealing it and by its persistence, even at 37° C., on serial transfer. One strain put through 25 transfers on agar and in broth in July and August retained throughout its production of the rich golden-brown color.

The organism is an unencapsulated, Gram-negative, non-sporing, motile rod with the characteristic shape, size and arrangement of coliform bacteria. Acid and gas are produced in mediums containing dextrose, lactose, salicin, galactose, and mannite, but not saccharose, dulcite, cellobiose, alpha-methyl-d-glucoside, inosite, raffinose, inulin, or adonite. Litmus-milk is acidified and coagulated as if by *B. coli communis*. H_2S is not produced nor is gelatin liquefied. Indol is formed from tryptophane, the methyl-red reaction is positive whereas the Voges-Proskauer is negative and citrate does not furnish a suitable source of carbon for the growth of the organism.

Bergey (4th Ed.) lists 22 species of *Escherichia*. Of these the chromogenic strain resembles only *Escherichia paragruenthali* and it differs from this recognized species not only in being chromogenic but in failing to ferment raffinose. Following Jordan (11th

Ed.) we prefer to speak of all *coli-aërogenes* organisms as *Bacterium* instead of *Escherichia* (the *coli*) and *Aërobacter* (the *aërogenes*). For the strain described the name *Bacterium aurescens* is therefore proposed.

The serum of a rabbit immunized with *Bact. aurescens* reacted with the homologous strain to a limit of 1:2560, with agglutination complete at 1:640. This serum did not give distinct reactions with any available similar but non-chromogenic coliform cultures. The strain failed to react distinctly with 2 of 3 anti-coli serums at hand. With the third it gave a complete reaction at 1:320. Unfortunately the exact titre for this serum with its engendering strain is not on record. In a group so antigenically heterogeneous such data suffice only to show distinct difference on the one hand and relationship on the other.

Cruickshank[5] placed *Bact. typhi flavum* in the genus *Chromobacterium*. *Bact. aurescens* is an organism whose habitat, biochemical characters and colonial appearance are definitely those of coliform bacteria. None of the 68 species of *Flavobacterium* or of the 10 species of *Chromobacterium* listed by Bergey closely resemble the organism here described.

The original saline suspension prepared January 14 was stored in the cold room. When plated 248 days later the only lactose-fermenting aërobic bacteria recovered were chromogenic *coli* possessing the same biological characteristics as the original *Bact. aurescens*.

We[6] have shown that where an original fecal suspension contains only *coli*, as far as *coli-aërogenes* organisms are concerned, no amount of storage will reveal any other lactose-fermenting aërobic organisms than *coli*. On the other hand, if the original specimen contained *coli* and *aërogenes* or *coli-aërogenes* intermediates, even though these non-*coli* forms be present in such small numbers as to be missed in the original plating, storage in the cold results in their increase at the expense of *coli* to such an extent that plating then reveals them. This fecal specimen, therefore, probably contained a pure culture (disregarding anaërobes, etc.) of *Bact. aurescens*.

This fact and the commonly current belief, with its sanitary implications, that chromogenic *coli* are non-fecal, lead me to describe the organism and report its occurrence.

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[5] Cruickshank, *J. Hygiene*, 1935, **35**, 354.

[6] Parr, *Am. J. Pub. Health*, 1936, **26**, 39.

9055

Diurnal Variation in the Free Activity of Sheep and Pig.

Quan F. Curtis* (Introduced by H. S. Liddell.)

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In the investigation of the problem of experimental neurosis in animals, one of the possible causative factors to be considered is restriction through habituation of the animal's freedom of movement. As an approach to this part of the problem, a study of the animal's spontaneous activity previous to any restriction becomes pertinent.

Experimental neurosis in the sheep leads to a permanent state of hyperexcitability with loss of formerly established discriminations.[1] In the dog the neurosis may manifest itself in chronic somnolence or in hyperexcitability during the conditioning tests.[2] It is reasonable to suppose that the absence of the enduring somnolent or inhibitory experimental neurosis in the sheep may be related to the fact that the sheep does not sleep as the dog does. The pig is a sound sleeper and for this reason it was decided to compare the diurnal cycles of spontaneous activity in these sleeping and non-sleeping animals.

The subjects for investigation were 10 sheep, of both sexes, ranging in age from 1½ to 8 years, and 5 pigs from the same litter, 14 months old, 3 females and 2 males, one castrate. The sheep and male pigs ran in a 15 acre pasture while the sows were limited to a one-acre field. Each of the animals wore a standard New Haven pedometer attached to a harness. They had all become thoroughly habituated to the harness before any records were taken. The pedometers were read twice daily, at 6 A. M. and 6 P. M. Readings were continued for a period of 16 days, from October 15 to October 31. At this season of the year, the intervals between readings corresponded closely to the actual hours of daylight and darkness.

The pedometers were calibrated at the beginning and end of the study by determining the number of pendulum movements per recorded mile for each. The table shows the average amount of

* National Research Fellow in the Biological Sciences.

[1] Anderson, O. D., and Liddell, H. S., *Arch. Neurol. and Psychiat.*, 1935, **34**, 330.

[2] Pavlov, I. P., *Conditioned Reflexes*, London, 1927.

activity (steps taken) by the sheep and pigs during 24 hours, and the division of this activity between day and night.

	Day		Night	
		%		%
Sheep	2629	(78)	714	(22)
Pig	3130	(88)	386	(12)

The ratio of day to night activity for the pigs is more than twice that for the sheep. How strongly the pig's free activity is conditioned upon daylight is shown by this observation: at the beginning of the experiment when there was light at both 6 A. M. and 6 P. M. the pigs were found moving about the yard at these hours; but toward the end, when it was dark both morning and night, the pigs always had to be roused from sleep.

Two incidental influences upon free activity may be noted. In certain of the female animals there was an opportunity during the course of the experiment to observe the effect of the oestrous cycle. For example, one sow during heat ran 8700 steps in 24 hours as contrasted with an average of 4001 steps for 10 other 24-hour periods when not in heat. A striking manifestation of the sow's hyperactivity was her tendency to run about at night: in the night of the selected 24-hour period during heat she took 2243 steps as contrasted with an average of 346 for 10 other nights. The weather also seemed to have some effect on the activity of the animals. During the 2 coldest nights the sheep and pigs' activity was reduced by nearly 25%.

9056

Studies in Sympatheticomimicity, III: Physiological Effects of More Non-amino Catechol Derivatives.

RAYMOND L. OSBORNE. (Introduced by F. B. Flinn.)

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Muhlmann[1] was the first to point out that catechol (3,4-dihydroxy-benzene) produced a rise in the blood pressure of the intact anesthetized rabbit. Dakin[2] attributed much of the typical action of epinephrine to the catechol nucleus. Later, Barger and Dale[3]

[1] Muhlmann, *Deutsch. Med. Wochnsch.*, 1896, **22**, 409.

[2] Dakin, *Roy. Soc. Proc. B.*, 1905, **76**, 498.

[3] Barger and Dale, *J. Physiol.*, 1910, **41**, 19.

showed that non-catecholic amines produced similar effects: they attributed the action of sympatheticomimetics to the amine group and drew the conclusion that catechol raises the blood pressure by direct muscle stimulation, rather than by sympathetic imitation. Mulinos and Osborne[4, 5] demonstrated that catechol, ethylcatechol (3,4-dihydroxyphenylethane), acetocatechol (alpha(3,4-dihydroxyphenyl)ethanone), and chloracetocatechol (alpha(3,4-dihydroxyphenyl)betachloroethanone) possess sympatheticomimetic properties in which the inhibitor effects predominate. Mulinos and Osborne[4] claimed that the catechol nucleus is in great part responsible for the typical sympatheticomimicity of epinephrine, and that the 3,4-position of the phenyl hydroxyls, a 2-carbon sidechain, a beta-carbon hydroxylated, an alpha-carbon hydrogen substituted by some indifferent radical, preferably an amine, and with all of these the levorotatory isomer, were the factors in the formation of a sympatheticomimetic drug.

Weinstein and Manning[6] stated that monomethylamino-3:4-quinone and monomethylaminoethanol-3:4-quinone, which are adrenalone and epinephrine respectively with the phenyl hydroxyls converted to the quinone form, have no effect upon the blood pressure. Thus destruction of the catechol nucleus has obliterated the vasopressor power, even though the amine and skeleton of epinephrine and adrenalone are still present.

In this paper we report the effects of 6 new compounds upon the blood pressure of the cat. Various modifications of the alpha(3,4-dihydroxyphenyl)ethanone skeleton were synthesized. We have introduced one, 2 or 3 acetyl radicals into the frame in the 3- or 4-hydroxy, 3- and 4-hydroxy positions of the phenyl group, and in both hydroxys and on the beta-carbon of the side chain. We introduced a hydroxy in place of the chlorine in chloracetocatechol. And we introduced one and 2 acetyls in the catechol nucleus.

The compounds thus synthesized were: alpha(3,4-acetoxy-hydroxyphenyl)betachloroethanone, alpha(3,4-diacetoxyphenyl)betachloroethanone, alpha(3,4-diacetoxyphenyl)betaacetoxyethanone, alpha(3,4-dihydroxyphenyl)betahydroxyethanone, 3,4-diacetoxybenzene, and 3,4-acetoxy-hydroxybenzene.

These drugs were tested for blood pressure effects in 10 cats, using epinephrine as a control. No other drugs were injected other than those described in this paper. Chloracetocatechol and catechol were tested in 48 cats. Doses of 2.5 to 10 mg. were given intra-

[4] Mulinos and Osborne, PROC. SOC. EXP. BIOL. AND MED., 1935, **32**, 1344.

[5] Mulinos and Osborne, PROC. SOC. EXP. BIOL. AND MED., 1935, **33**, 458.

[6] Weinstein and Manning, PROC. SOC. EXP. BIOL. AND MED., 1935, **32**, 1096.

venously in the intact cat anesthetized with intraperitoneal nembutal, 40 mg. per kilo, and atropinized with 2 mg. per kilo of the sulfate intramuscularly; both vagi were cut. The rises in pressure varied from 25 to 50 mm. of mercury, depending upon the dosage and drug. Figures 1, 2 and 3 show typical effects for 3 different types of drugs. From this it is seen that acetylation of the phenyl-hydroxyls does not eradicate the vasopressor power of the alpha (3,4-dihydroxyphenyl)ethanone group, nor of catechol; the blood pressure rise is in no way diminished, if not increased by acetylation.

Conclusion. Ten non-amine catechol derivatives cause a rise in the blood pressure of the intact cat. Acetylation of catechol and the

FIG. 1.

FIG. 2.

FIG. 1.

Effect of alpha (3,4-diacetoxyphenyl) betaacetoxyethanone, 5 mg., on blood pressure (A) and respirations (B) of the intact cat. Neutral solution. All figures reduced four times.

FIG. 2.

Effect on blood pressure and respirations of 10 mg. of alpha (3,4-acetoxy-hydroxyphenyl) betachloroethanone; solution neutral to litmus.

FIG. 3.
Effect of 3,4-diacetoxybenzene on the blood pressure and respiration; 10 mg. were given.

alpha(3,4-dihydroxyphenyl)ethanone derivatives does not destroy the vasopressor ability. The physiological effects of these drugs on other organs and the effects on the blood pressure of the pithed, decerebrated and demedullated cat are now being studied and will be reported in the near future.

9057

Effect of Epicaine on Blood Pressure.

RAYMOND L. OSBORNE. (Introduced by F. B. Flinn.)

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We[1] reported the discovery of a new drug, a vasopressor local anesthetic: alpha(3,4-dihydroxyphenyl)beta(paraaminobenzoylbetadiethylaminoethanol)alphaethanonehydrochloride, designated for brevity as *epicaine*.

Procaine and its known relatives cause a pronounced fall in blood pressure, as does cocaine, in the ordinary concentrations.[2-5] Epicaine raises the blood pressure in the intact cat.

We[6] have previously pointed out that biochemorphologically the following factors are contributory to the formation of a typically sympatheticomimetic drug: the 3,4-position of the phenyl hydroxyls, a 2-carbon sidechain, a beta-carbon hydroxylated, an alpha-carbon hydrogen substituted by some indifferent radical, preferably an amine (but not necessarily so), and the laevorotatory isomer. The chemical structural configuration of epicaine is compatible with these criteria.

The drug was injected into intact cats anesthetized with pentobarbital (40 mg. per kilo). Ten cats were used; in 8 both vagi were cut and the animal given atropine sulfate intramuscularly (2 mg. per kilo) at the start of the experiment. In 2 animals ether was used and the cats were neither atropinized nor vagotomized. Thirty-eight injections of the drug were given, and each was always controlled by epinephrine. The cannula was washed after each injection until no effect was obtained from 2 successive washes before the next injection of a drug.

Immediately upon intravenous injection of epicaine in doses from 2 to 10 mg. there is an abrupt rise in blood pressure ranging from about 20 to 40 mm. of mercury. This is illustrated in Fig. 1. Concomitant with the increase in pressure there is a dilatation of the pupil and a retraction of the nictitating membrane. Subsequent doses continue to elicit the same effect.

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1 Osborne, R. L., *Science*, 1937, **85**, 105.

2 Osborne, R. L. (to be published).

3 Roth, *Hyg. Lab. Bull.*, 1917, **109**

4 Roth, *J. Pharm. and Exp. Ther.*, 1917, **9**, 352.

5 v. Anrep, *Arch. ges. Physiol.*, 1880, **21**, 38.

6 Mulinos and Osborne, PROC. SOC. EXP. BIOL. AND MED., 1935, **32**, 1344.

FIG. 1.
Five mg. of epicaine were injected at arrow. Initial pressure was 195 mm. of mercury. EC is epicaine. Figure reduced four times.

We[7] have stated elsewhere that in the intact animal ergot reversal and cocaine synergy of a blood pressure rise indicate that the impulses eliciting the rise reach the blood vessels by way of the sympathetic nerves; they do not indicate the site of origin of the impulses. Therefore these invalid criteria were not employed; further experiments are now being carried out on decapitated, decerebrated, and demedullated, as well as pithed, animals.

[7] Mulinos and Osborne, PROC. SOC. EXP. BIOL. AND MED., 1935, **33**, 458.

9058

Pituitary Acidophile Cells After Excision of the Superior Cervical Sympathetic Ganglia in the Rat.

ISOLDE T. ZECKWER. (Introduced by E. B. Krumbhaar.)

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Studies of the pituitary following thyroidectomy in young rats[1] have been reported, describing the appearance of large numbers of basophilic cells containing hyaline material, as a constant feature in all rats in which thyroidectomy was adequate, as evidenced by retardation of body growth, of kidney growth and alterations in the ratios of gonads and adrenals to kidney growth.[2] During these experiments a puzzling feature was the variation in the acidophile loss. In some cretin pituitaries, all acidophiles had disappeared, while in others there was a moderate or slight reduction in number. It was thought that perhaps a factor in the disappearance of the acidophiles might be some incidental trauma to nerves in the course of the thyroidectomy,[3] as recent work had suggested a nervous control of the anterior pituitary through the cervical sympathetic nerves. Collin and Hennequin[4] had found in male adult rabbits that ablation of one of the superior cervical sympathetic ganglia resulted in vasodilation of the pituitary and a rapid loss of acidophiles in 5 hours to 7 days. On the 7th day the pituitary was composed chiefly of chromophobes. Stimulation of the central end of the cervical sympathetic caused an increase in the granules and numbers of acidophiles in one-half to three-quarter hours. That nervous stimulation of the pituitary may occur is indicated by the fact that Friedgood and Pincus[5] found that stimulation of the cervical sympathetic nerves in adult female rabbits resulted in extensive maturation of the ova and in some cases ovulation.

In performing thyroidectomy in young rats the cervical sympathetic fibres could be traumatized inadvertently, as the thyroid is bound laterally closely to the carotid artery region. The superior cervical sympathetic ganglia lie high above the level of the thyroid and deep beneath the carotid artery so that direct trauma to them in

[1] Zeckwer, I. T., Davison, L. W., Keller, T. B., and Livingood, C. S., II, *Am. J. Med. Sci.*, 1935, **190**, 145.

[2] Zeckwer, I. T., Proc. Am. Physiol. Soc., *Am. J. Physiol.*, 1936, **116**, 166.

[3] Zeckwer, I. T., Proc. Phila. Physiol. Soc., *Am. J. Med. Sci.*, 1936, **191**, 872.

[4] Collin, R., and Hennequin, L., *Compt. rend. Soc. de biol.*, 1936, **121**, 81.

[5] Friedgood, H. B., and Pincus, G., *Endocrinology*, 1935, **19**, 710.

the course of thyroidectomy would be impossible. To be sure, occasional rats showed closure of one eye, but this had been thought due to inflammation rather than to section of cervical sympathetic fibres. In order to rule out the possibility that trauma to cervical sympathetic fibres at the level of the thyroid was a factor in our experiments, sympathetic nerves in rats were severed with and without thyroidectomy. Since the fine nerve fibres are difficult to identify with certainty in young rats, in a number of experiments one or both superior cervical sympathetic ganglia were easily identified and removed so as to interrupt the fibres with certainty. The consequent permanent almost complete closure of the corresponding eye and enophthalmus proved the adequacy of the operation. In some experiments the region near the carotid arteries was stripped of all fibres except the vagus. In other operations laryngeal nerves were severed. Both sexes of rats were used, 54 varying in age from 28 to 39 days, and 9 from 65 to 96 days of age. The operations included the following: (1) bilateral excision of superior cervical sympathetic ganglia without thyroidectomy in 10 rats; (2) bilateral excision of superior cervical sympathetic ganglia with thyroidectomy in 5 rats; (3) excision of one superior sympathetic ganglion in 7 rats; (4) excision of one superior cervical sympathetic ganglion with thyroidectomy in 1 rat; (5) both recurrent laryngeal nerves cut in 4 rats; (6) both recurrent laryngeal nerves cut with thyroidectomy in 5 rats; (7) other nerve trauma in neck in 17 rats; (8) the same with thyroidectomy in 3 rats; and (9) as controls, thyroidectomy without trauma to nerves in 11 rats which were littermates of the others and were killed at varying time intervals corresponding to the rats with nerve trauma. Intervals after operation of 1 to 34 days were studied.

In no case did severance of nerves without thyroidectomy result in any extensive loss of acidophiles. Counts were not made but this loss after thyroidectomy is so obvious that no counts were necessary. After trauma with thyroidectomy, the pituitary presented exactly the same histological picture as after thyroidectomy without trauma. In order to permit legitimate interpretation of loss of acidophiles, histological sections of several different pituitaries were always mounted on the same slide, so that they would be stained in an identical manner with Mallory's acid fuchsin anilin blue orange G stain after fixation in Helly's fluid. In one cat experiment, also, unilateral excision of one superior cervical sympathetic ganglion resulted in no obvious loss of acidophiles in 33 days.

Species differences in endocrine reactions makes it unwise to assume that the findings of one species will apply to another. The

FIG. 1.

A representative field of the pituitary of a rat 4 days after bilateral excision of superior cervical sympathetic ganglia showing a normal number of acidophile cells. (a) acidophile, (b) basophile, (c) chromophobe. The smallest darkest cells are acidophiles, and constitute about 50% of all cells in the field.

experiments of Collin and Hennequin were on rabbits and these animals differ from others in such endocrine reactions as their manner of ovulation. The changes in the rabbit pituitary following thyroidectomy, as described by Bryant[6] and Marine, Rosen and Spark,[7] are quite different from the changes we observe constantly in rats, cats and dogs. The time intervals of the present experiments were in many cases longer than those of Collin and Hennequin because we are concerned only with long continued changes in relation to thyroidectomy.

Summary. Excision of superior cervical sympathetic ganglia and severance of other nerves in white rats did not result in any obvious decrease of acidophiles in the pituitary in 1 to 34 days. When such operations were combined with thyroidectomy, the changes in the pituitary were exactly the same as the changes caused by thyroidectomy alone.

Variations in the degree of acidophile loss after thyroidectomy in rats, therefore, can not be attributed to nerve trauma.

[6] Bryant, A. R., *Anat. Record*, 1930, **47**, 131.

[7] Marine, D., Rosen, S. H., and Spark, C., PROC. SOC. EXP. BIOL. AND MED., 1934, **32**, 803.

9059

Cytotoxic Glomerular Nephritis in Rabbits—a Non-Allergic Condition.

W. E. Ehrich. (Introduced by E. B. Krumbhaar.)

From the Department of Pathology, School of Medicine, University of Pennsylvania.

The important discovery of Masugi[1] that in rabbits and rats glomerular nephritis can be produced by intravenous injections of anti-kidney serum has been confirmed by Fahr[2] and his pupils Hemprich[3] and Weiss,[4] in rabbits; by Arnott, Kellar and Matthew,[5] in rabbits; and by Farr and Smadel,[6, 7] in rats. It has been shown that the animals develop albuminuria (up to 30%, Esbach), hematuria, cylindruria, lipuria, moderate oliguria or occasionally anuria (in acute stages), moderate edema, rise in blood urea (up to 224 mg. %), rise in blood pressure, cardiac hypertrophy, and anemia, and that at autopsy the kidneys exhibit extensive glomerular lesions which closely resemble those of human glomerular nephritis.

Masugi injected 10 cc. of a 10 to 30% suspension of blood-free kidney juice of rabbits in saline solution into the peritoneal cavity of ducks, and that 18 to 30 times at intervals of about 5 days. Five to 8 days after the last injection serum was prepared, and after inactivation by heating to 56° C. for 30 minutes, was injected intravenously once or repeatedly into rabbits in doses of 5 to 15 cc. Most rabbits received more than 15 cc. of serum. Hemprich suspended the juice of 2 kidneys in 100 cc. of saline solution. Of this suspension, 10 cc. were injected into ducks 9 to 22 times at 4 to 8 days' intervals. The serum of these animals was injected into rabbits in doses of 3.5 to 26 cc. Most rabbits received more than 10 cc. of serum. Weiss improved the method by thoroughly squeezing the remnants of the ground up kidneys while preparing the juice. He produced glomerular nephritis already with doses of 3 to 7 cc. of ducks' serum, in most cases with 4 to 5 cc. Arnott, Kellar and Mat-

1 Masugi, M., *Beitr. Path. Anat.*, 1933, **91** 82; 1934, **92**, 429.

2 Fahr, Th., *Verh. Deutsch. Path. Ges.*, 1935, **28**, 179; *Klin. Wchnschr.*, 1936, **15**, 505.

3 Hemprich, R., *Z. ges. exp. Med.*, 1935, **95**, 304.

4 Weiss, A., *Beitr. Path. Anat.*, 1935, **96**, 111.

5 Arnott, W. M., Kellar, R. J., and Matthew, G. D., *Edinburgh Med. J.*, 1936, **43**, 217.

6 Farr, L. E., and Smadel, J. E., *J. Clin. Invest.*, 1936, **15**, 449.

7 Smadel, J. E., *Am. J. Path.*, 1936, **12**, 742.

TABLE I.
The Production of Glomerulonephritis by Antikidney Serum in Rabbits.

Preparation of Serum					Production of Glomerulonephritis					
Duck No.	Duck weight (gm.)	Dose of kidney juice (cc.)	No. of doses given	No. of days treated	Rabbit No.	Rabbit weight (gm.)	Dose of serum (cc.)	Killed days after injection	Glomerulo-nephritis	No. of Glomeruli affected %
1	2400	5	16	60	93	1550	7	93	+	13
2	2450	5	20	77	36	1880	3.5	47	+	82
					34	2000	7	Died within 24 hours		
4	2270	10	16	60	94	1560	3.5	63	+	84
					42*	1540	5.0	14	+	68
					96	1480	7.0	Died within 24 hours		
3	2750	10	20	77	35	1660	3.5	63	+	8
					33	2040	7.0	47	+	42

*Injected with a serum which was preserved by the lyophile process of Mudd (*J. Immunol.*, 1935, **29**, 389), and kept for 80 days.

thews, using the original method of Masugi, injected the ducks 25 to 40 times.

Since the experimental production of glomerular nephritis is an actual problem, additional information may be welcome. It may be permitted, therefore, to present the following preliminary data:

The kidneys of a freshly killed rabbit were perfused *in situ* with sterile saline solution until they appeared to be free of blood. Then they were ground in a mortar and the juice thus prepared was diluted with 60 cc. of sterile saline solution. After centrifugation, the ground kidney remnants were thoroughly squeezed with 2 forceps, and their juice added to the supernatant fluid of the centrifugate. The suspension then was injected intraperitoneally into Peking ducks in doses of 5 or 10 cc. at intervals of 4 to 5 days for 60 to 77 days (16 to 20 injections). Four days after the last injection the ducks were decapitated, the blood collected, and the serum inactivated by heating to 56° C. for 30 minutes. The serum then was injected intravenously into rabbits in doses of 3.5 to 7 cc., each rabbit receiving one injection only.

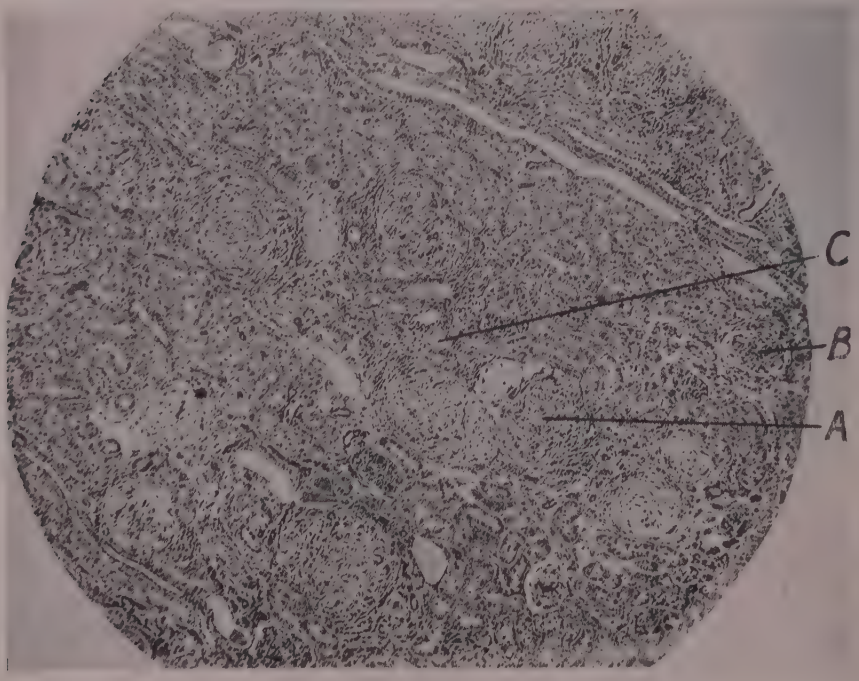

FIG. 1.

Experimental glomerular nephritis. Hematoxylin-Eosin, × 70. Most glomeruli are much enlarged and badly damaged (A); some appear to be normal (B); many tubules contain casts (C).

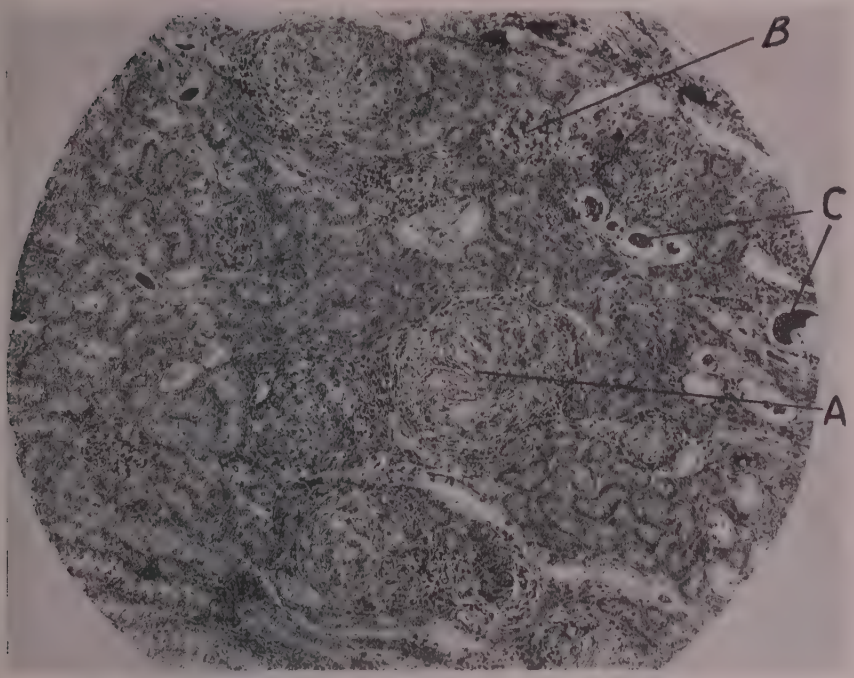

FIG. 2.
Experimental glomerular nephritis. Azur II, × 100. For description see Fig. 1.

The results of the experiments are given in Table I. With the exception of 2 rabbits which died within 24 hours after the injection, all rabbits developed glomerular nephritis. The larger the dose injected and the more powerful the serum, the greater the number of glomeruli affected. That the histological changes closely resemble those of human glomerular nephritis, is shown in Figures 1 and 2. The clinical findings were: albuminuria, oliguria, temporary rise in blood urea, temporary anemia and edema. The urinary sediment and the blood pressure were not studied.

Concerning the nature of the nephritis produced, it should be noted that the lesions were focal in all instances. As compared with human glomerular nephritis, the changes resembled not so much those of diffuse hemorrhagic glomerular nephritis as those of focal glomerular nephritis as observed in bacterial endocarditis. Concerning the etiology of our disease, it may be emphasized that all the rabbits received one injection only. This glomerular nephritis, therefore, can not be looked upon as an allergic disease, as done by Masugi and others; it rather should be classified as a toxic disease, the toxin being a specific antibody, a so-called cytotoxin.

9060

Box for Observation of Living Organs *in situ* and for Abdominal Surgery in the Sculpin.

Allan L. Grafflin and M. J. Eisenberg. (Introduced by W. deB. MacNider.)

From the Department of Anatomy, Harvard Medical School.

The operating box herein described was developed for use in 2 studies upon the sculpin (*Myoxocephalus octodecimspinosus*), a common marine teleost. Grafflin and Ennis[1] reported experiments upon the blockage of the gastrointestinal tract, and Grafflin and Eisenberg[2] upon the direct observation of the living kidney. There is obviously a wide field for comparative studies in the fishes in which abdominal surgical procedures and direct observation of living organs *in situ* would play an important part. The sculpin is readily available for experimental work; and the operating box which we have developed has been so satisfactory in our experience that it is described here in the belief that it will prove useful to other workers. The box is illustrated in Figures 1 and 2.

With the use of water outlets C and C′, the water level in the box is usually maintained at a sufficiently high level. With some larger specimens a higher level is necessary to insure complete covering of the head, and in these cases the rubber tubing is switched from C to D, and the opening at C is plugged. The rather tight fit of the animal between the clamps, and the upward projection of the pectoral fins, suffice to maintain a higher level in the anterior than in the posterior chamber under these conditions.

A constant stream of sea water is supplied through the inlet B. In the experiments previously reported urethane anesthesia was used. Anesthesia was rapidly induced by immersing the fish in a 1% solution of urethane in sea water in a separate chamber. The animal was then transferred to the box, and anesthesia was maintained throughout the experiment by a 0.25% solution of urethane in sea water. This was supplied by gravity from a large container, the flow being sufficiently rapid to effect a constant change of water in the box. The solution becomes somewhat contaminated in passing through the box, and should not be used a second time. If the anesthesia becomes too light, the clamps and lead blocks usually suffice to prevent disturbing movement until the concentration can be adjusted. The angle at which the pectoral fins are held and the

[1] Grafflin, A. L., and Ennis, D., *J. Cell. and Comp. Physiol.*, 1934, **4**, 283.

[2] Grafflin, A. L., and Eisenberg, M. J., *Anat. Rec.*, 1934, **59**, 449.

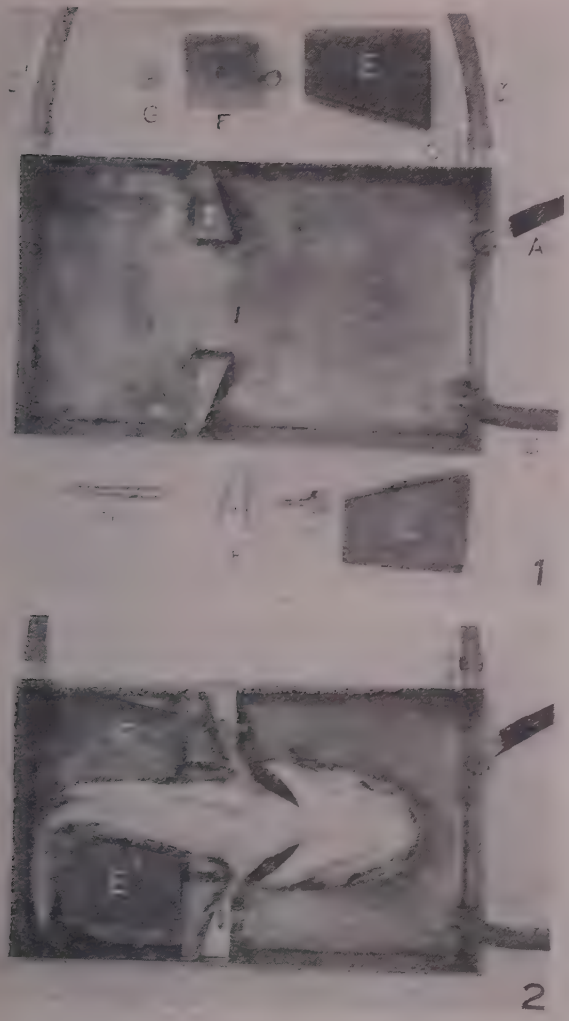

FIG. 1.
Operating box, unassembled, approximately × ⅓
FIG. 2.
Operating box, assembled, with the sculpin in position for operation.

The over-all dimensions are: length 7⅜", width 4⅛", height 2⅝". The sides were constructed of 2" stock brass, the ends of sheet brass, all ⅛" stock, and the joints were soldered. The box and all accessory parts were then nickel-plated to prevent corrosion by sea water.

A—air inlet.

B—water inlet; the tube is held in a spring metal clamp.

C, C'—water outlets at the same level, the center of the lumen being 18 mm. below the top of the box.

D—accessory water outlet, with the center of the lumen 12 mm. below the top of the box. C, C' and D are of ¼" brass tubing.

E, E′—heavy lead blocks for stabilization of posterior portion of fish. Length 5 cm., lesser width 2.6 cm., greater width 4 cm., thickness 2.4 cm.
F, F′—removable portions of pectoral fin clamps. Note the two projecting pins, which fit snugly into holes in the posts I, I′.
G, G′—screws for holding blocks F, F′ in position.
H—key for tightening screws G, G′. Key fits into small holes in screw head (see G′).
I, I′—permanent portions of pectoral fin clamps, attached by screws to bottom and sides of box. The complete pectoral fin clamp for one side (F and I) is made by sawing at an angle through a block of brass metal 30 mm. long, 19 mm. wide and 32.5 mm. high. The relative dimensions of the two portions are sufficiently indicated.

relatively large size of the anterior chamber allow perfect freedom of respiratory movements. It was found desirable to aerate the anterior chamber, and this procedure should be followed in all experiments.

In ordinary abdominal surgical procedures in the sculpin, it is preferable to have the operative field exposed by suitable retractors. However, if an assistant is not available, the box is so constructed that excellent exposure can be readily obtained, and the walls of the incision held sufficiently high to prevent the entrance of sea water into the abdominal cavity.

On careful examination of Figures 1 and 2 one can see a row of small holes closely spaced around the top of the box. These holes are 2 mm. in diameter, and their centers are 4 mm. below the upper edge of the sides. After the incision is made, ties are taken through the margins of the wound, and the threads are carried through various of these holes depending upon the exposure desired. After the proper tension has been exerted upon the threads, they are held so by inserting tapered, round wooden pegs into the holes. This simple manner of retraction was found to be invaluable in the experiments upon direct observation of the kidney. The box adapted itself readily to such experiments in which the Leitz Ultropak Microscope was used.

The sculpin has been extensively used for investigative work over a period of years, and, in the group of readily available teleosts, seems to be a rather ideal experimental animal. It is a skin fish with a yielding abdominal wall, adapting readily to ordinary surgical procedures. It is relatively resistant to asphyxia and anesthesia, and could be used with little difficulty for experiments involving prolonged microscopic observation of living organs *in situ*.

9061 P

Avitaminosis. XVIII. Peripheral Nerves in Vitamin B_1 Deficiency as Observed by Polarized Light.*

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Although the literature is full of reports on nerve degeneration in avian polyneuritis and B deficiency in the rat and other mammals, the most recent contributions in this field reveal conflicting evidence.[1] In order to circumvent uncertainties in the interpretation of Marchi preparations and any possible artefacts that may be introduced during fixation and staining, Sutton, Setterfield and Krauss[2] have recently applied the polarized light technique for the study of degeneration of myelinated nerves in vitamin A deficiency. This technique was found by Setterfield and Baird[3] to have advantages over the Marchi method of staining with osmic acid, since it does not depend upon fixation and staining but on the chemical structure of the myelin sheath; moreover, it is more rapid, more sensitive and constant.

The polarized light technique, however, as employed by Sutton and associates requires previous fixation of tissues in formalin, which procedure may introduce artefacts. We have applied the technique of Sutton and co-workers in studies of vitamin B deficiency and have checked the formalin procedure by elimination of all fixatives as well as all staining operations. Small pieces of spinal cord, trigeminal, sciatic and optic nerves from avitaminotic and litter mate controls on the same plane of nutrition, were fixed in 95% alcohol in liquid air, and dehydrated *in vacuo*, at −32°C., by the Altmann-Gersh-freezing-drying technique, as modified by Scott and Williams.[4] Frozen sections were made and compared under polarized light with those fixed in 10% formalin. Twelve groups of rats were used for this comparative study. The pathological animals of 9 groups were on diets deficient only in vitamin B_1 and had varying degrees of polyneuritis, ranging from slight to marked paralysis. The avitaminotic animals of the rest of the groups

* Research paper No. 432, Journal series, University of Arkansas.

1 Prickett, C. O., *Am. J. Physiol.*, 1934, **107**, 459; Zimmerman, H. M., *Arch. Path.*, 1932, **13**, 207; Duncan, D., *Arch. Neurol. and Psychiat.*, 1931, **25**, 327.

2 Sutton, T. S., Setterfield, H. E., and Krauss, W. E., *Ohio Agr. Exp. Sta. Bull.*, No. 545, Dec., 1934.

3 Setterfield, H. E., and Baird, T. T., *Stain Technology*, 1936, **11**, 41.

4 Scott, G. H., and Williams, P. S., *Anat. Rec.*, 1936, **66**, 475.

were on a diet deficient only in the vitamin B complex. No demonstrable differences were observed by the 2 methods of study. We are now certain that the formalin fixation, previous to the use of polarized light, is quite a dependable procedure.

In addition to the above, we have studied, to date, 2 polyneuritic and 3 complex groups, involving a total of 32 animals, 16 pathological and an equal number of controls. The most regular and marked myelin degeneration was found in the sciatic and trigeminal nerves. About 50% of the avitaminotic animals showed a slight degeneration of the spinal cord. The optic nerves were normal in all pathological animals suffering from either polyneuritis or a deficiency of the vitamin B complex. The extent of loss of weight had no direct relationship to the severity of nerve degeneration. Nerve degeneration has been observed in animals that did not develop paralysis on the vitamin B complex deficient diet.

9062 P

Bisulphite Binding Substances in the Blood in Health and in Disease, Particularly Vitamin B_1 Deficiency.

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From the Thorndike Memorial Laboratory, Second and Fourth Medical Services (Harvard), Boston City Hospital, and the Department of Medicine, Harvard Medical School.

Bisulphite binding substances (B.B.S.) in the blood have been reported increased in vitamin B_1 deficiencies both in experimental animals[1] and in clinical "wet beriberi".[2] The present study of the B.B.S. in the blood was undertaken to determine its value in distinguishing vitamin B_1 deficiencies from other disease states.

Oxalated blood samples were taken fasting and at rest from 30 healthy controls, and from 110 patients. The method of Clift and Cook[3] was modified to adapt its use to trichloracetic acid filtrates of whole blood. Five milliliters of oxalated blood were precipitated with 20 ml. of 10% trichloracetic acid, allowed to stand 30 minutes and centrifuged. Five milliliter aliquots of the supernatant liquid were adjusted to pH 2 by the addition of 1.5 ml. of normal sodium

[1] Thompson, R. H. S., and Johnson, R. E., *Biochem. J.*, 1935, **29**, 694.
[2] Platt, B. S., and Lu, G. D., *Quart. J. Med.*, 1936, **5**, 355.
[3] Clift, F. P., and Cook, R. P., *Biochem. J.*, 1932, **26**, 1788.

hydroxide and allowed to react with 0.2 ml. of saturated sodium bisulphite solution for 15 minutes. Twenty-five milliliters of distilled water and 2 ml. of fresh starch solution were added and the excess of bisulphite titrated out with normal and N/10 iodine solutions adjusting the end point with N/200 iodine solution and N/100 sodium thiosulphate solution. The bound bisulphite was released by the addition of 2 gm. of solid disodium phosphate ($Na_2HPO_4 \cdot 12\ H_2O$) and titrated within 5 minutes with N/200 iodine solution. The values were expressed in milligrams of pyruvic acid per 100 ml. of blood, using the expression: 1 ml. N/200 iodine 0.22 mg. pyruvic acid. In most cases the non-protein nitrogen, the plasma carbon dioxide capacity and blood sugar were measured on the same samples. In a smaller group the urine was tested for sugar, acetone, acetoacetic acid and pyruvic acid.

Results. Thirty normal subjects had values for B.B.S. in the blood ranging between 3.66 and 5.75 mg. with an average of 4.74 mg. per 100 ml. Values for B.B.S. above 6.0 mg. are accepted as elevated. Normal values were found in the following conditions: arteriosclerosis, compensated heart disease, carcinomatosis, glomerulonephritis, hepatitis with jaundice, severe anemia, toxemia of pregnancy, acute alcoholism, chronic alcoholism without vitamin deficiency, chronic alcoholism with vitamin (B_1) deficiency after treatment. Increase in the B.B.S was found in the following diseases: decompensated heart disease, unregulated diabetes, vascular nephritis, lobar pneumonia and certain other febrile diseases, chronic alcoholism with vitamin (B_1) deficiency untreated, terminal uremia, pancreatitis, and pernicious vomiting. Among the factors usually associated with an elevation in the B.B.S. were acidosis, ketosis, anoxemia, "toxemia" and uremia. In certain cases of "vitamin B_1 deficiency" and of diabetes, however, the elevation in the B.B.S. could not be related to these factors. In both of these conditions abnormal amounts of pyruvic acid were found in the urine in a few of the cases in which it was studied. Whether or not the elevation of the B.B.S. in these conditions represents an elevation also of the pyruvic acid in the blood awaits further observations.

Conclusions. An elevation of the B.B.S. in the blood is found in a variety of different diseases, including vitamin B_1 deficiencies. Since vitamin B_1 deficiencies rarely occur without the possible complication of one or more of the other diseases in which there may be an elevation of the B.B.S., we have not found the B.B.S. in the blood of aid in diagnosing vitamin B_1 deficiency.

9063

Inactivation of Pneumococcal Hemolysin by Certain Sterols.*

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From the Johns Hopkins Medical School, Baltimore.

It is well known that cholesterol is an effective inhibitor of bacterial hemolysins and saponins; but with the possible exception of the saponins[1] the chemical mechanism of the inhibition is obscure. So far as concerns bacterial hemolysins, the problem is more complex and confused because it is not even certain that the lysins from different bacterial species belong to one chemically defined class of compounds. This situation can be clarified only after systematic studies, of which our examination of pneumococcal hemolysin forms a part.

We have already demonstrated that the lysin can be reversibly oxidized by a variety of agents, and have indicated that its activity is associated with the presence of the thiol grouping in the lysin molecule.[2] In view of the strong inhibitive action of cholesterol, we turned next to an exploration of the effects of this and certain other sterols in an endeavor to learn more concerning the chemistry of the lysin. As will be seen, our present findings indicate, among other things, that the hemolytic activity of this enzyme-like substance is conditioned, in addition, by a second grouping.

Experimental. The hemolysin was an M/15 phosphate (pH 7.6) extract of washed, frozen and thawed pneumococcus (type II) cells; and our hemolytic unit was that amount of extract which would completely hemolyze 2 ml. of a 1 volume percent suspension (pH 7.6) of thrice washed rabbit red cells.

Each sterol suspension was made by dissolving 5 mg. of the compound in 5 ml. absolute methanol, which was then poured slowly into 100 ml. boiling water. This stock 0.00013 M suspension was filtered to remove some small flakes which separated out (these were usually scarcely weighable in amount); it was then cooled and diluted with water for use. In some cases the suspensions were made without boiling. The higher concentrations required for cholesteryl-acetate were obtained by using the methanol solution direct, along with suitable controls to rule out the effect of solvent. The fresh

* Under the term sterol, we have included for convenience both the alcohols and the derivatives.

[1] Windaus, A., *Nachr. Kgl. Gesellsch.* Göttingen, 1916-17, p. 301.

[2] Shwachman, H., Heilbrunn, L., and Cohen, B., *J. Biol. Chem.*, 1934, **107**, 257.

stock suspensions were of fairly uniform nephelometric density; and occasional colorimetric, gravimetric and nephelometric determinations were made to check and adjust the concentrations of the stock preparations. Nevertheless, the uncertainties involved in the handling of these suspensions were such that it is well to remember that the inhibiting doses reported below represent generally the maximum calculated quantities.

The procedure followed was to allow graded quantities of the sterol (in 0.1 ml. volume) to act on 1 hemolytic unit (in 0.1 ml.) of the lysin for 20 to 30 min., after which red cells were added and the resulting hemolytic activity determined as usual. The minimum quantity of sterol which inactivated one unit was taken as the inhibiting dose.

It may be stated that the minimum inhibiting doses were reproducible and of the same relative magnitudes under any one set of experimental conditions. The variations from time to time were due to uncontrollable differences in the sterol suspensions, the various hemolysin extracts, and the red cells. The ranges given in Table I are the more instructive in that they show the effects obtained under diverse conditions.

The experiments to be reported first were made upon fully active hemolysin. (For present purposes, we consider the lysin to occur in 2 forms: active and inactive. The fully active form is the natural product protected from mild air-oxidation; the fully inactive form is the air-oxidized product, which can be restored to activity by treatment with cysteine or H_2S). The results given in Table I show the high inhibiting potency of cholesterol, and the relative potencies

TABLE I.
Inhibiting Effect of Sterols on Active Pneumococcal Hemolysin.

Common name	Chemical name (a)	Digitonin precipitability	Amount required to inhibit one hemolytic unit (mg.)
Cholesterol (b)		+	0.00001-0.0001
Cholesteryl acetate (c)		—	over 0.2
Allocholesterol	Coprostenol (d) (e)	+	0.00001-0.0001
Coprosterol	Coprostanol (d)	+	0.01-0.05
Cholestenone	Coprostenone (d)	—	0.1-0.3
Pseudocholestene	Coprostene (d)	—	0.1

(a) Nomenclature according to Rosenheim and King, *J. Soc. Chem. Ind.*, 1934, **53**, 91.

(b) Eastman product twice converted to the dibromide and thrice recrystallized from abs. ethanol. Method of Schönheimer, *Z. physiol. Chem.*, 1930, **192**, 86.

(c) Eastman product.

(d) Kindly supplied by Dr. Rudolf Schönheimer.

(e) Prepared by the Windaus method (m.p. 117°), and recently shown by Schönheimer and Evans, *J. Biol. Chem.*, 1936, **114**, 567, to contain admixed cholesterol.

of the other sterols examined. It may be of interest to note that, if cholesterol and lysin react mole for mole, the concentrations of lysin in the test solutions were of the order of 10^{-6} to 10^{-7} molar. This inhibition of the fully active hemolysin is irreversible in the sense that subsequent treatment with cysteine or H_2S does not restore any of the original lytic activity.[3]

Table I shows that the digitonin precipitable sterols in the series inhibit the lysin in much smaller doses than do the digitonin-negative. (The chemical names of the sterols disclose chemical and structural differences not evident from the common names.) It is seen that the unsaturated alcohols, cholesterol (cholestenol) and coprostenol, differing only in the position of the double bond, are equal in inhibiting potency. (The recently discovered fact that our sample of coprostenol contained admixed cholesterol should not alter this interpretation.) On the other hand, saturation of the double bond as in coprostanol is accompanied by a considerable diminution of inhibiting potency. The digitonin-negative sterol derivatives, all unsaturated, were far less effective as inhibitors. We are inclined to ascribe the fact that some of them did inhibit in much higher concentrations to an effect different from that under discussion.

These findings on pneumolysin are in general harmony with the old observations on tetanolysin[4, 5] and on saponin[6]; and they furnish, in addition, more quantitative comparisons with probably better defined compounds than were then available.

In contrast to the above effects on active lysin, we find that the oxidized, inactive form is influenced by cholesterol very much more slowly, if at all. For example, when a unit of inactive lysin is treated with an inhibiting dose of cholesterol for 30 min. at 37°, the hemolytic activity can be restored almost completely by subsequent treatment of the mixture with cysteine or H_2S. (Here, the quickly regenerated lysin was determined before the cholesterol had a chance to exert its somewhat slower effect.) On the other hand, active lysin similarly treated shows no restoration, except occasionally to a small degree, doubtless due to small amounts of admixed inactive form. A known mixture of the two forms behaves in accordance with this view.

[3] Our suggestion (*J. Bact.*, 1936, **31**, 67) that the inhibitive effect of cholesterol can be reversed was based on results with lysins that turned out to be partly inactive.

[4] Abderhalden, E., and LeCount, E. R., *Z. exp. Path. Therap.*, 1906, **2**, 199.

[5] Walbum, L. E., *Z. Immunitätsf.*, 1910, **7**, 544.

[6] Hausmann, W., *Hofmeisters Beitr.*, 1905, **6**, 567.

It seems reasonable to conclude therefore that the state of oxidation of the lysin conditions its reactivity with cholesterol; and that the presence of the OH group in the sterol molecule is apparently essential for the reaction. It is possible that the spatial configuration of the effective group or groups (as indicated by the parallelism with the digitonin reaction of the compounds under discussion) is also a determining influence; but this will require further study.

Effect of cholesterol on the nitroprusside reaction. The ordinary active hemolysin extract (containing 50 to 150 hemolytic units per ml.) is usually too dilute to give a positive reaction to a nitroprusside test capable of detecting at least 1×10^{-7} mole of cysteine in 0.5 ml. volume. In more concentrated extracts (of about 250 units per ml.) a positive reaction of an intensity corresponding to about 5×10^{-7} mole of cysteine can be obtained. Since a number of quasi-specific reactions[2] of the lysin point to the association of a thiol grouping with its action on the red cell, it becomes of interest to determine if the inhibiting action of cholesterol affects the availability of this grouping. We find that it apparently does not; for a nitroprusside-positive extract treated with 5 to 10 times the inhibiting dose of cholesterol for 15 min. still remained nitroprusside positive. As would be expected, a parallel experiment with cysteine and cholesterol gave the same result.

It would seem, therefore, that the inactivation caused by cholesterol leaves the SH group of the lysin free; in other words, that the point of attachment (or attack) on the lysin molecule by cholesterol is somewhere else than on the SH group. Consequently, it is reasonable to infer that the lytic activity is associated with at least 2 different groupings: one capable of reversible oxidation and reduction, and the other more or less specific for a certain type of sterol grouping and configuration.

That these functional groupings are not independent has been indicated above and is supported by the following observations. As is well known, the active lysin is taken up quickly and practically entirely by an equivalent amount of red cells. We find, on the other hand, that the inactive form is adsorbed little if at all under similar conditions. For example, 4 ml. red cell suspension were put into each of 2 centrifuge tubes. To tube 1 was added 0.1 ml. inactive extract containing about 2 potential hemolytic units; to tube 2 was added 0.1 ml. phosphate buffer. The mixtures were incubated at 30° for 25 min. and then spun out. The supernatant fluid from tube 1 was then added to the sedimented cells of tube 2, and *vice versa*. The cells were resuspended, and 0.1 ml. of 0.06 M cysteine was added to each suspension, after which the tubes were incubated.

The final result was as follows: tube 1 showed no sign of hemolysis; tube 2 showed 85% hemolysis, equal to that in a third, control tube containing lysin, red cells and cysteine in the same proportions.

These results, taken together with the fact that much free cholesterol occurs in the red cell, may mean that the red cell sterol attaches active pneumococcal lysin as an antecedent to hemolysis. But we are not yet prepared to accept this view without more conclusive data. It is, however, of interest in this connection that cholesterol-treated red cells remain vulnerable to the hemolysin, although the rate of hemolysis is retarded more or less.

The effect of contaminants (peroxides). The picture presented above seems fairly self-consistent; nevertheless, owing to the nature of our materials, it is necessary to consider the possibility of another factor, *viz.*, peroxide formation, as a source of significant error in interpretation. This arises from 2 related facts. In the first place, hemolysin exposed to H_2O_2 is affected rather rapidly. Secondly, cholesterol is known to undergo spontaneous oxidation in air,[7] a process which apparently involves a peroxide stage, for we find a week-old stock 0.00013 M suspension of the purified sterol to give a pronounced test for peroxide with ferrous thiocyanate reagent[8] and a weaker test with acidified KI. The freshly made suspension is negative, and in addition shows antioxidant action by greatly retarding the spontaneous oxidation of the thiocyanate reagent.

Granting these facts, does peroxide formation account for the inhibiting action of cholesterol? Our observation that cholesterolized lysin retains its free SH grouping is one point against such a view. We find, however, that the sterol, pretreated with a crude catalase preparation, retains some 80 to 90% of its inhibiting potency. This, taken along with the fact that traces of peroxide can be detected in saturated solutions of our cholesterol in methanol (although not detectable in the highly diluted suspensions actually employed) would indicate that only a small part of the inhibition can be assigned to peroxides.

It is hoped that concentrations of the lysin now in progress will provide material better suited for analysis of the various aspects of the problems here touched upon.

Summary. I. It is shown that the inhibiting effect on pneumolysin by cholesterol and certain related sterols is apparently determined primarily by the presence of the OH group in the sterol struc-

[7] Schulze, E., and Winterstein, E., *Z. physiol. Chem.*, 1904-5, **43**, 316.

[8] Young, C. A., Vogt, R. R., and Nieuwland, J. A., *Ind. Eng. Chem., Anal. Ed.*, 1936, **8**, 198.

ture and secondarily by the double bond. Possible peroxide formation would seem to account for only a small portion of the cholesterol effect. 2. Active pneumolysin is inhibited promptly, while inactive (air-oxidized) lysin is affected slowly if at all by cholesterol. Likewise, active lysin is adsorbed by red cells, while the inactive form is not. That is, the state of oxidation of the lysin conditions its reactivity with the sterol and with its adsorption on red cells. 3. The free thiol grouping on the active lysin molecule remains free after treatment with excess cholesterol. The lytic activity, therefore, is associated with at least 2 functional groupings, one reversibly oxidizable, and the other more or less specific for a certain sterol grouping and configuration.

9064 P

Synthesis of Protein and Amino Acids in Mice with the Aid of Deuterium.

JAKOB A. STEKOL AND WILLIAM H. HAMILL.

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Schoenheimer and Rittenberg[1] have proposed that if non-labile deuterium is found in the molecule of a fatty acid isolated from tissues of mice which were receiving heavy water, this finding is then indicative of the synthesis of the fatty acid in the animal body. In connection with the study of the synthesis of protein and amino acids in animals, we were interested to ascertain whether or not a similar criterion, as used by Schoenheimer and Rittenberg, is applicable to protein and amino acid synthesis. Barbour, *et al.*,[2] on the assumption that one hour after a single injection of D_2O into mice a maximum of exchangeable deuterium will be fixed in the tissue, conclude that inasmuch as "mice drinking 15% D_2O for 2 months have 3 times the concentration of deuterium (relative to body water deuterium) in the tissue as mice receiving a single injection of D_2O," the difference in the deuterium content of the tissue indicates the fixation of deuterium in tissue in stable form. It occurred to us that the isolation of tissue protein and of amino acids derived therefrom and the analysis of the isolated products from which all deuterium in labile position has been removed would constitute more direct evidence for the fixation of deuterium in the

[1] Schoenheimer, R., and Rittenberg, D., *J. Biol. Chem.*, 1935, **111**, 163.

[2] Smith, P. K., Trace, J., and Barbour, H. G., *J. Biol. Chem.*, 1936, **116**, 371.

protein in stable form than the one offered by Barbour.[2] Twenty adult and 20 growing mice were used in a preliminary study. The animals were kept, each group separately, in metabolism cages and 2% D_2O and food consisting of 70 parts of oats, 15 of yeast powder and 15 of "Klim" milk powder were allowed *ad libitum*. A record of food and water consumption of the young mice was taken daily. The young mice were weighed every 3 to 5 days, while the weight of the adult mice was taken at the beginning and at the end of the experiment. Beginning on the 3rd day of maintenance on the 2% D_2O, a mouse was withdrawn from the group of growing mice, killed by a blow, and the body fluid and protein isolated. This was done thereafter every 3-5 days, in order to ascertain the changes in the deuterium content of the body fluid and of the protein with growth. After 10 days, the group of adult mice was killed, body fluid removed by distillation *in vacuo* with the aid of dry ice, and the protein isolated in the same manner as in the case of growing animals. The method of Addis, *et al.*, was employed.[3] The protein thus isolated was then digested with HCl or H_2SO_4 in the usual manner and various amino acids were isolated and identified by analysis. The deuterium content of the body fluids, protein and the amino acids was estimated as follows: Samples of protein or of amino acids to be analyzed, after treatment to ensure complete removal of deuterium in labile positions by repeated precipitation from acid and alkali, were dried *in vacuo* and then burned in the regular manner according to Pregl. The combustion water was collected by condensation in a small glass tube packed in dry ice. To ensure complete removal of excess deuterium from the furnace, about 30 mg. of urea were burned in 2 portions and these flushings combined with water first obtained. This combustion procedure has been tested by analysis of known compounds and found quantitatively correct. The water so obtained was diluted by weighed addition of tap water to bring the total to about 60-70 mg. Then this water was heated in a sealed tube at 140°C. with solid NaOH and $KMnO_4$ for 18 hours, then distilled *in vacuo* repeatedly. The density of the purified water was determined with a small diver (weight, 2 mg.), regulated by temperature and sensitive to a temperature difference of 0.01°C. or 0.002% D_2O. Divers were made of quartz and were calibrated by reference to purified tap water. The diver was observed by means of a cathetometer, the containing vessel being immersed in a glass-walled thermostat which maintained constant temperature within 0.01°C.

[3] Addis, T., Poo, W. J., Lew, W., and Yuen, D. W., *J. Biol. Chem.*, 1936, **113**, 497.

TABLE I.
The Deuterium Content of Protein, Amino Acids and Body Fluid of Mice which received 2% D_2O.

Animal	Adult		Non-Adult			
Days on D_2O	0	10	3	6	9	14
Weight, gm.			9.0	11.3	13.1	15.1
Body fluid, *% D_2O		1.03	0.74	0.96	1.00	1.20
Protein, *% D_2O	0.002	0.100	0.105	0.118	0.179	0.200
l-cystine, *% D_2O		0.230				
Tyrosine, *% D_2O		0.290				

*% D_2O represents excess of D_2O with reference to tap water.

Some of the typical results are shown in Table I. The data seem to indicate that the protein of both the adult and growing mice receiving D_2O contains deuterium in a non-labile form. The isolation of deuterium-containing amino acids from the protein of adult mice further confirms this observation. The protein similarly isolated from control mice receiving distilled water and the same diet was found to contain deuterium in concentrations not exceeding those usually found in tap water. The deuterium content of the protein isolated from the growing mice ran parallel to the gain in weight of mice, suggesting synthesis of protein with growth. The isolation of the known amino acids from the protein of the growing mice which received D_2O is now in progress. We hope by further study of the manner in which deuterium could possibly enter the molecule of an amino acid to throw additional light on the rôle of amino acids in the protein synthesis from the standpoint of their indispensability in nutrition. Should the assumption that the presence of deuterium in an amino acid in non-labile form is an indication of the synthesis of this amino acid in the animal body be correct, our finding as regards l-cystine and tyrosine indicates that these amino acids can be synthesized by the adult mouse.

9065

Absence of Effect of Swallowed Saliva on Coagulation of Blood.

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It has been shown[1, 2] that saliva added to shed blood has a remarkable accelerating action both on normal and haemophilic bloods. It was thought desirable to see if saliva, when swallowed, had any effect on the coagulating time of blood or contributed in any way to its coagulability. Mills[3, 4] has shown that the ingestion of a meal rich in protein has an accelerating effect on the coagulation of blood but the ingestion of fats or carbohydrates has little effect.

The results of this work may be briefly stated. It is easy to confirm Mills' observations. Thus a meal of 600 gm. of meat caused a reduction of clotting time from 3¼ minutes to 1 minute 54 seconds two hours after the meal. No, or at most a few seconds, reduction in clotting time resulted from carbohydrate or fat meals and this is true also for the ingestion of large amounts of saliva. Paraffin or gum was chewed for 15 to 30 minutes and the saliva swallowed as formed. Samples of blood were taken every 15 minutes and in no case was any material difference in clotting time noted. Thus the average of 6 experiments carried out on 6 different individuals shows a clotting time before of 3 minutes 14 seconds, one hour after the saliva had been swallowed it was 3 minutes 15 seconds. The capillary tube method of Mills and Petersen[5] was used for obtaining the coagulation times.

In one dog all the salivary glands were removed aseptically and although the animal was kept a number of months the coagulating time of its blood remained normal.

Thus no effect on the coagulating time of blood could be made out either from an increase or a decrease in the amount of saliva swallowed. It might be pointed out that cobra and other snake venoms which affect coagulation when injected into the blood are without effect when taken by the mouth

[1] Bellis, C. J., Birnbaum, W., and Scott, F. H., Proc. Soc. Exp. Biol. and Med., 1932, **29**, 1107.

[2] Bellis, C. J., and Scott, F. H., Proc. Soc. Exp. Biol. and Med., 1933, **30**, 1373.

[3] Mills, C. A., *J. Biol. Chem.*, 1923, **18**, 55.

[4] Mills, C. A., and Necheles, H., *Chinese J. Phys.*, 1928, **2**, 219.

[5] Mills, C. A., and Petersen, M. F., *Arch. Int. Med.*, 1923, **32**, 188.

9066 P

Excretion of Radio-Sodium Following Intravenous Administration in Man.

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After the first extensive internal use of naturally radioactive compounds in man by Proescher and others,[1-5] Seil, *et al.*,[6] estimated that from 40% to 60% was lost from the body, chiefly through the feces. A progressive diminution in the rate of excretion was indicated by Schlundt and his group,[7] who found that patients retained 4.3% 6 months after administration and 1.9% 6 months later. Schlundt and Failla[8] found 24 μgm. and 14 μgm. respectively in 2 women 12 years after oral ingestion of radium, with respective coefficients of excretion of .005% and .0025%. Prolonged retention has been reported in many cases of industrial radium poisoning.[9, 10]

Following the announcement by Curie and Joliot[11] of the preparation of an artificial radioactive isotope of nitrogen in 1934, Lawrence and his co-workers[12] produced relatively large amounts of radioactive isotopes of many elements using their magnetic resonance accelerator. Radio-sodium became available for clinical study at the University of California Hospital in the Spring of 1936. It was felt that many of the disadvantages of internal radium therapy could be avoided by the use of radio-sodium, since this latter substance does not tend to become fixed in the body tissues and the

1 Proescher, F., *Radium*, 1914, **2**, 61.

2 Delano, S., *Radium*, 1915, **6**, 1.

3 Proescher, F., and Almquist, B. R., *Radium*, 1916, **6**, 85.

4 Arneth, *Berl. klin. Wchnschr.*, 1914, **51**, 153.

5 Hirschfeld, H., and Meidner, S., *Z. f. klin. Med.*, 1913, **77**, 407.

6 Seil, H. A., Viol, C. H., and Gordon, M. A., *Radium*, 1915, **5**, 40.

7 Schlundt, H., Neraney, J. T., and Morris, J. T., *Am. J. Roentgenology and Radium Therapy*, 1933, **30**, 515.

8 Schlundt, H., and Failla, G., *Am. J. Roentgenology and Radium Therapy*, 1931, **26**, 265.

9 Martland, H. S., *Am. J. Cancer*, 1925, **15**, 2435.

10 Flinn, F. B., *Arch. of Phys. Ther., X-ray and Radium*, 1932, **13**, 476.

11 Curie, I., and Joliot, F., *Nature*, 1934, **133**, 201.

12 Henderson, M. C., Livingston, M. S., and Lawrence, E. O., *Phys. Rev.*, 1934, **45**, 428.

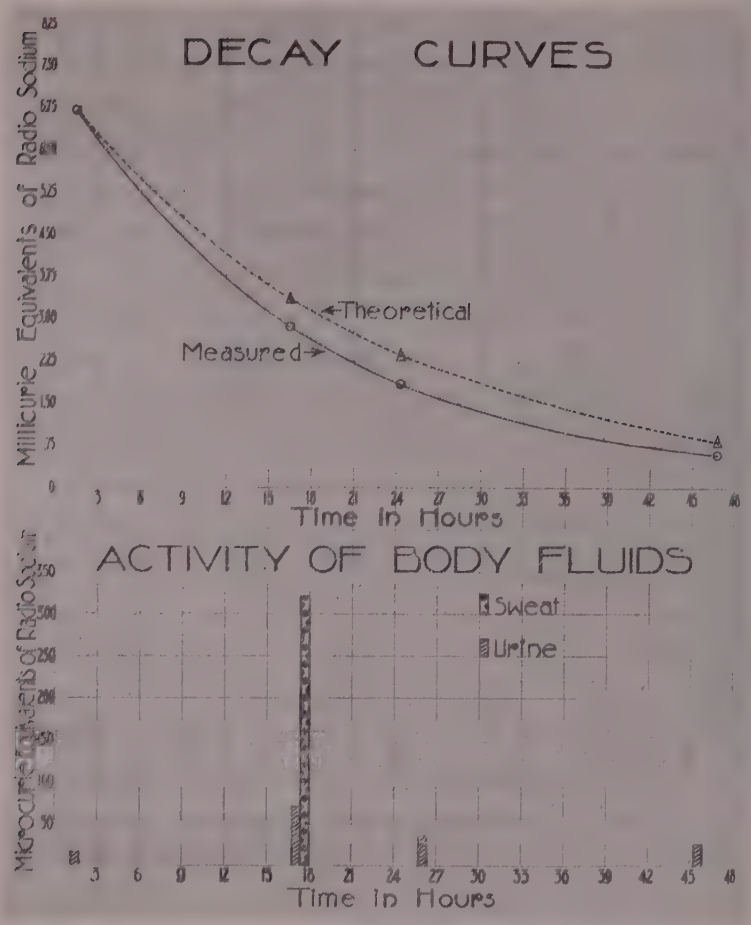

FIG. 1.

duration of its effect is limited by its short half-life of only 14.8 hours.

Initial investigations were made of the clinical effect and the rate of excretion of the radio-sodium following its intravenous administration to 2 human leukemic subjects.* An approximately isotonic solution of sodium chloride was used in each experiment.

* We are grateful to Prof. E. O. Lawrence and his associates in the Radiation Laboratory of the University of California, Berkeley, for furnishing the radio-sodium and for advice in regard to physical measurements, and to Drs. S. R. Mettier and E. H. Falconer for clinical cooperation.

Each sample was measured with an electroscope just prior to administration and periodic determinations of the degree of activity of the patient's body was carried out. At the same time all the stool and urine samples were collected and their activities measured. The activity of 100 cc. of blood from the second patient was determined. The first patient received 13 mc.e. (milli-Curie equivalents) of radio-sodium and in Fig. 1 are shown the measured and theoretical decay curves in the upper portion of the chart. The theoretical values were computed from the first measurement of the patient's activity. The lower portion of each chart illustrates

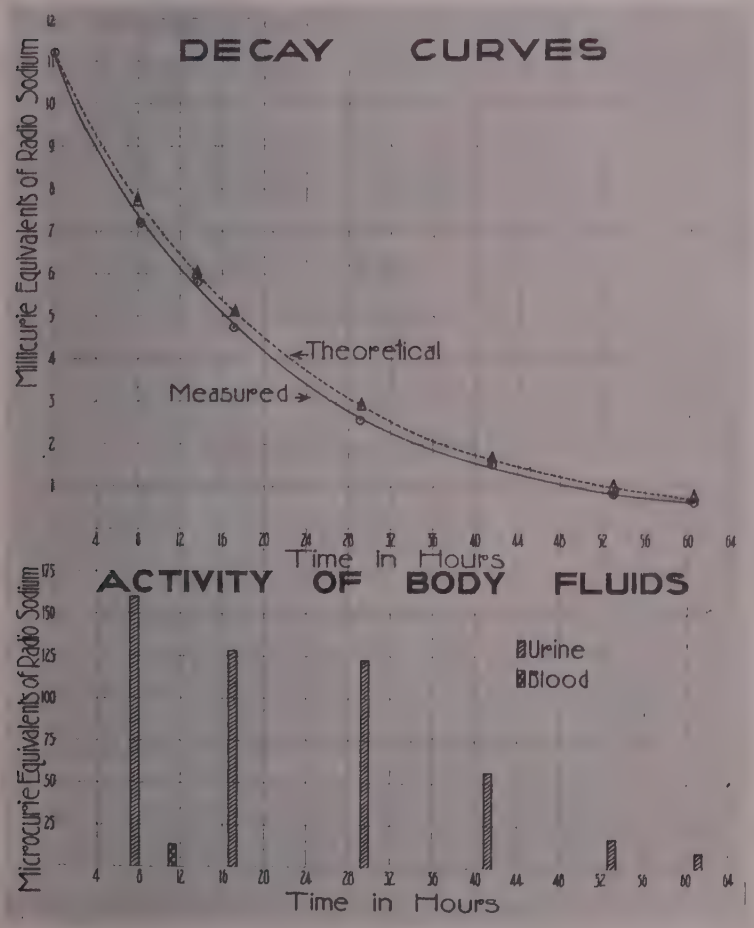

FIG. 2.

the amount of activity observed in the various body fluids. The values in Fig. 2 were observed following the administration of 15 mc.c. of radio sodium to the second patient. The abscissa indicates the intervals of time following injection, and the ordinates the degree of activity.

The difference between the activity of the sample of radio sodium just before administration and the initial activity measurement of the patient is felt to be due to the absorption of the gamma rays by the patient's body. In Fig. 1 it will be noted that a relatively large proportion of the radio sodium was eliminated in the sweat, while in the second case the activity of the sweat was too small to be determined. This can possibly be explained by the fact that the first patient had frequent drenching sweats.

The interval between the actual and theoretical decay curves in each experiment is felt to represent the quantity of radio-sodium lost through the various channels of elimination. This view is borne out by the fact that the quantities excreted by each subject correspond approximately to the difference between the two curves in Figs. 1 and 2. In each instance the activity of the feces was too feeble to be measured.

9067

Sedimentation of Erythrocytes in Solutions of Albumin, Fibrinogen and Peptone.*

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Fahraeus[1, 2] has shown that the most rapid sedimentation of erythrocytes takes place in solutions of fibrinogen, and the least rapid in solutions of albumin. He concluded that an increase in the serum globulin and fibrinogen "stands in direct causality" with the rapidity of sedimentation. Westergren[3, 4] stated that there exists a definite

* Aided by a grant from the Christine Breon Fund.

1 Fahraeus, R., *Acta Med. Scand.*, 1921, **55**, 1.

2 Fahraeus, R., *Physiol. Rev.*, 1929, **9**, 255.

3 Westergren, A., Theorell, H., and Widström, G., *Z. f. d. g. exp. Med.*, 1931, **75**, 668.

4 Westergren, A., Juhlin-Dannfelt, C., and Schnell, R., *Acta Med. Scand.*, 1932, **77**, 469.

positive correlation between the plasma or serum proteins and the rate of sedimentation of erythrocytes. Zárday and Farkas[5] modified normal whole blood by the addition of normal fibrinogen and globulin, and found that the sedimentation rate was increased in proportion to the amounts added. When, instead, they added normal albumin they found that the rate of sedimentation was decreased. Coburn and Kapp[6] reached similar conclusions and added that "albumin inhibited sedimentation almost completely."

Using a modification of the Linzenmeier[7] technique, Lucia and co-workers[8, 9, 10] found that, *in vitro*, a consistent relationship cannot be established between the sedimentation rate and the globulin content of various solutions of serum globulin. They also showed that, *in vivo*, the sedimenation rate is *apparently* accelerated as the globulin content of the serum increases, and is retarded as the albumin content increases; and that a cause-and-effect relationship between the quantity of the various serum proteins and the rate of sedimentation cannot be said to exist.

Groups of human subjects with normal and with rapid sedimentation rates were chosen for this study. From each patient a 20 cc. sample of venous blood was withdrawn and oxalated. A sedimentation test was done directly on this sample, using the Linzenmeier technique. The remainder of the sample was separated by centrifugalization, and the corpuscles washed with 3 changes of Locke's solution. Then 0.2 cc. of washed corpuscles were resuspended in 0.8 cc. of each of the following menstrua: plasma, Locke's solution, and varying concentrations of albumin, fibrinogen and peptone in plasma and in Locke's solution. The resulting hematocrit values were 20%, or 2.15 million corpuscles per cmm. All sedimentation experiments were done using the Friedlander tube and recording the time necessary for the column of erythrocytes to drop 18 mm. The albumin and fibrinogen were prepared by electrodialysis.† In all resuspension experiments the syringes were rinsed in 10% solution of potassium oxalate.

[5] Zárday, I., and Farkas, G., *Z. f. d. g. exp. Med.*, 1931, **78**, 367.

[6] Coburn, A. F., and Kapp, E. M., *J. Clin. Invest.*, 1936, **15**, 715.

[7] Linzenmeier, G., *Arch. f. Gynaekologie*, 1920, **113**, 608.

[8] Lucia, S. P., and Brown, J. W., PROC. SOC. EXP. BIOL. AND MED., 1934, **32**, 189.

[9] Lucia, S. P., Gospe, S. M., and Brown, J. W., PROC. SOC. EXP. BIOL. AND MED., 1935, **33**, 356.

[10] Lucia, S. P., Blumberg, T., Brown, J. W., and Gospe, S. M., *Am. J. Med. Sci.*, 1936, **192**, 179.

† This material was prepared by Dr. D. M. Greenberg of the Department of Biochemistry.

In 2 experiments in which a solution of beef-blood serum-albumin was suspended in Locke's solution, in percentages varying from ½ to 3, the sedimentation time was either unaltered or slightly prolonged as compared with a control. A portion of the same albumin solution suspended in plasma also retarded the sedimentation time progressively as the content of albumin increased, and in direct proportion to the increased volume of diluent added to the plasma.

In 2 experiments in which a solution of human blood serum-albumin was suspended in Locke's solution, the sedimentation time was prolonged regardless of the concentration of albumin. The same product suspended in plasma markedly and progressively prolonged the sedimentation time as the quantity of albumin was increased. These experiments were corrected for the degree of dilution of the plasma.

In 4 experiments, in which powdered electrodialyzed human blood-serum-albumin was dissolved in Locke's solution, in percentages varying from 1 to 5, sedimentation time was progressively and significantly shortened as the quantity of albumin was increased. In 4 experiments in which the same product was dissolved in plasma, it prolonged the sedimentation time as the quantity of albumin was increased.

In 5 experiments in which a 1% solution of beef-blood serum-fibrinogen was suspended in Locke's solution in concentrations varying from 0.2 to 1.0%, the sedimentation time was unaltered. In 2 of 5 experiments in which the same product was suspended in plasma, the sedimentation time appeared to be prolonged though not to a significant degree.

In 2 experiments in which Witte's peptone was suspended in Locke's solution in concentrations varying from 1 to 10%, the sedimentation time was prolonged irregularly but not significantly. In 2 experiments in which this product was suspended in plasma, the sedimentation time was prolonged but not to the same degree as it was in the controls in which plain Locke's solution was used.

In several experiments, talc or kaolin suspended in plasma failed to alter the sedimentation time.

In 3 experiments in which dog-bile in concentration of 1:50 to 1:3,200 was suspended in plasma, the sedimentation time was prolonged as the concentration of the bile increased.

Summary. Beef-blood serum-albumin, when suspended in Locke's solution or in plasma, prolonged the sedimentation time of human erythrocytes. A solution of human-blood serum-albumin suspended in Locke's solution and in plasma prolonged the sedi-

mentation time. A sample of powdered human-blood serum-albumin shortened the sedimentation time when it was suspended in Locke's solution, and prolonged it when suspended in plasma. A solution of beef-blood serum-fibrinogen suspended in Locke's solution and in plasma did not significantly affect the sedimentation time. Witte's peptone suspended in Locke's solution or in plasma prolonged the sedimentation time insignificantly. Talc or kaolin were without effect on the sedimentation time. Dog-bile suspended in plasma prolonged the sedimentation time.

9068 P

Propylene Glycol: Rate of Disappearance from the Blood Stream.

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The increasing use of propylene glycol as a solvent for various pharmaceutical preparations[1] and coloring extracts,[2] and its possible use as a food, indicates the need for a better understanding of the actions of this alcohol. The known production of oxalic acid in the metabolism of ethylene glycol[3, 4] has been responsible for the substitution of propylene glycol for the former, since oxalic acid is not a possible metabolite of the latter.[5] The properties, general actions, and acute and chronic toxicities of propylene glycol have been investigated by Seidenfeld and Hanzlik[5]; toxicity was unusually low.

The concentration of propylene glycol in the blood was determined by treating the protein-free filtrate with a solution of potassium dichromate in strong sulphuric acid, and estimating the amount of reduced dichromate iodometrically. From this value was subtracted the amount of oxidizable material normally present in the blood, a variable which remains relatively constant in fasting animals. In urine, the glycol was estimated directly by using an aliquot portion

1 Brown, C. L. M., *Quart. J. Pharm. Pharmacol.*, 1935, **8**, 390.

2 Rae, J., *Pharm. J.*, 1935, **135**, 539.

3 Hunt, R., *Ind. Eng. Chem.*, 1932, **24**, 361.

4 Hanzlik, P. J., Seidenfeld, M. A., and Johnson, C. C., *J. Pharm. Exp. Therap.*, 1930, **41**, 387.

5 Seidenfeld, M. A., and Hanzlik, P. J., *J. Pharm. Exp. Therap.*, 1932, **44**, 109.

of a 1:100 dilution, and subtracting the blank value for normal urine from the final result.

The concentration of propylene glycol in the bloods of 2 dogs and 10 rabbits was followed for variable periods after giving doses from 1 to 12 cc. per kilogram body weight, intravenously or orally; the dogs were used repeatedly.

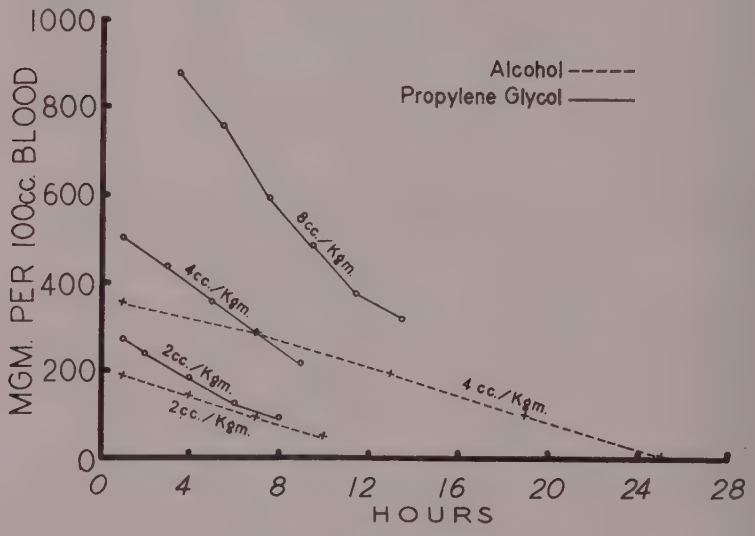

FIG. 1.

The rate of disappearance of propylene glycol from blood of dogs as compared with alcohol.

Figure 1 shows the rate of disappearance of propylene glycol from the blood of dogs following the administration of 2 cc. and 4 cc. per kilo intravenously, and 8 cc. gastrically; the intravenous doses are compared with similar doses of ethyl alcohol.

The rate of disappearance of propylene glycol from the blood is proportional to its concentration in the body, as contrasted with alcohol, which disappears at a constant rate.[6] This difference may possibly be accounted for by excretion of the propylene glycol by the kidneys, since it was possible to recover one-half the amount injected from the urine.

The concentration of propylene glycol in the blood after gastric administration indicates rapid absorption of the compound. Once it has entered the blood stream, it escapes rapidly and apparently diffuses throughout the body.

[6] Newman, H. W., and Cutting, W. C., *J. Pharm. Exp. Therap.*, 1936, **57**, 388.

The depressant effect is much less than that of alcohol. About 1100 mg. % of propylene glycol in the blood was required to attain the same degree of narcosis produced by a blood-alcohol concentration of 350 mg. %. The results on rabbits were essentially similar to those on dogs.

9069 P

A Graphic Representation of Thyroid Response to Stimulation by Thyrotropic Hormone.

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This is a report of a method of studying the hypertrophy of the thyroid gland in response to stimulation by the hypophyseal thyrotropic hormone. Other methods in use are: a study of the histological picture as used by Junkman and Schoeller[1] and by Aron[2]; Collip's method of determining the metabolic rate of the test animal[3]; McCullagh's method of determining the iodine content of the thyroid gland[4]; and Loeb's method of estimating the mitotic index of the thyroid.[5] During the past year Dr. Earl Blanck[6] has at the suggestion of Dr. Paul Starr, been making micrometric studies of the acinar cell heights of surgically removed thyroids, and correlating the micrometric histology with the clinical picture. Our method is based on the same principle and is analogous to the Price-Jones[7] blood curve.

As test animals we use female guinea pigs which have been kept on a standard laboratory diet and weigh from 180 to 225 gm. The procedure consists of administering 3 daily subcutaneous injections of Antuitrin T. The preparation was prepared and furnished us by Parke, Davis and Co. In this study we have used daily dosages corresponding to 0.0025 cc., 0.005 cc., 0.01 cc. and 0.02 cc. On the

1 Junkman and Schoeller, *Klin. Wchsft.*, 1932, **11**, 1177.

2 Aron, *Rev. franc. Endocrin.*, 1930, **8**, 472.

3 Collip and Anderson, *J. A. M. A.*, 1935, **104**, 965.

4 McCullagh, *J. Pharm. and Exp. Therap.*, 1935, **57**, 49.

5 Loeb and Kippen, *J. Pharm. and Exp. Therap.*, 1935, **54**, 246.

6 Blanck, personal communication.

7 Price-Jones, *Red Blood Cell Diameters*, Oxford Medical Publications, London, 1933.

fourth day the animals are autopsied and the thyroids removed and fixed in formalin. Paraffin sections stained with hematoxylin and eosin are placed on microscopic slides with the long axis in either the horizontal or vertical position.

The actual study of the sections is carried out under oil immersion by means of an ocular micrometer. The gland is covered systematically from end to end and in parallel pathways by means of the mechanical stage until 200 acini have been studied. Each acinus in the path of crossing whose lumen is large enough to present a definite cell wall is included in the study. Interacinar cells are not studied. From each acinus the cell which is most representative of the average height of the cells of that particular acinus and is clear enough to be measured is chosen and its height measured. The cell heights are tabulated and a frequency curve graphed. From these findings the mode, mean, standard deviation and probable error of the mean are determined.

Measurements from control animals formed curves with modes at 3.75 micra. The mean cell heights from 16 controls ranged from 3.59 to 3.98 micra. After treatment with 0.0025 cc. the curves had modes at 4.50 micra. The mean cell heights of 12 such animals ranged from 4.5 to 4.8 micra. After treatment with 0.0025 cc. the modes were at 4.5 and 4.9 to 5.6 micra. After 0.01 cc. the modes were also at 4.5 and 6.0 micra but the mean cell heights of 5 animals ranged from 5.5 to 6.0 micra. After 0.02 cc. the modes were at 5.25 and 6.75 micra and the mean cell heights of 5 animals ranged from 6.1 to 6.4.

Serial sections of one control gland and of one gland stimulated with 0.02 cc. were prepared and studied. Curves of the first 5 sections of each series demonstrate the constancy of this method.

In our study the most significant observation was the increasing mean cell height of each increasing dosage. Accordingly 1600 cell heights from 8 control animals as well as from each of the groups treated with 0.0025 cc. and 0.005 cc. of the hypophyseal extract, and 1000 cell heights of 5 animals from each of the groups treated

TABLE I.
Comparison of Mean Cell Heights of Increasing Dosage Series.

	No. of Cells	No. of Animals	Mean Height	P.E.
1. Controls	1600	8	3.77	.015
2. .0025 cc. Ant. T.	1600	8	4.65	.018
3. .005 " "	1600	8	5.25	.02
4. .01 " "	1000	5	5.71	.029
5. .02 " "	1000	5	6.14	.028

with 0.01 cc. and 0.02 cc. were tabulated. From these tabulations the mean cell height of each group and its probable error were determined. (Table I.) It is readily seen that the results of each successive group are of statistical significance.

Conclusion. We have described a micrometric method of representing the thyrotropic effect on the thyroid gland of test animals. Since each increased dosage of the hormone caused a characteristic curve, this procedure affords a quantitative method of determining the effects of the thyrotropic hormone on the thyroid of the guinea pig.

9070

Can Injected Sulfur be Utilized by the Animal Organism?

ROBERT W. VIRTUE AND HOWARD H. BEARD.

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Several workers have recently reported increases in the cystine content of fingernails following injection of colloidal sulfur. Lewis and Lewis[1] using rats and Geiling[2] with adult mice observed no utilization of dietary elemental sulfur for growth. The following experiments were carried out in an attempt to ascertain whether the animal organism could utilize injected colloidal sulfur for growth or for production of cystine. Eleven groups of 3 young rats each were used. The 3 members of each group were of the same age and approximately the same weight, and all 33 rats had identical dietary histories. During these experiments the first rat of each group received a cystine-deficient diet (dextrin 46, casein 9, sucrose 15, lard 19, cod liver oil 5, Osborne-Mendel salt mixture 4, and agar 2%) *ad libitum*. Rat 2 of each group was limited to the amount of stock diet eaten by rat 1 the previous day, and was given an intraperitoneal injection of 12 mg. of colloidal sulfur (Sulfur-diasporal)* every other day. Rat 3 of each group was likewise limited to stock diet equivalent to that eaten by rat 1, but received a daily supplement of 20 mg. of l-cystine. Each of the 33 rats was given a vitamin pill daily which contained 80 mg. of Harris-Vitamin

[1] Lewis, G. T., and Lewis, H. B., *J. Biol. Chem.*, 1927, **74**, 515.

[2] Geiling, E. M. K., *J. Biol. Chem.*, 1917, **31**, 190.

* The authors wish to thank the Doak Chemical Company of Cleveland for the Sulfur-diasporal used in these experiments.

powder. Rats 1 and 3 of each group were given intraperitoneal injections of oil every other day that were equivalent volumetrically and calorifically to the oil which made up the vehicle for the colloidal sulfur injected into rat 2. After 3 weeks on the diets as described the animals were killed and their bodies and hides analyzed separately for cystine. The method used was an iodine titration carried out under the conditions described by Virtue and Lewis for determination of disulfide compounds in urine.[3] Hydrolysates of the hides required no decolorization, but kaolin was employed to decolorize body hydrolysates. Results of a typical group analysis, and the totals for the 33 rats are found in Table I.

TABLE I.
Effect of Injections of Colloidal Sulfur on Cystine Content of Rats.

		Typical Group				Total (11 groups)			
Rat No.	Intake	Original wt., gm.	Final wt., gm.	Cystine content, mg.		Original wt., gm.	Final wt., gm.	Cystine content, mg.	
1	Stock diet	45	44	Hide	66	512	546	Hide	836
				Body	18			Body	298
				Total	84			Total	1134
2	Stock plus colloidal S	46	44	Hide	41	516	508	Hide	773
				Body	16			Body	299
				Total	57			Total	1072
3	Stock plus cystine	45	55	Hide	113	509	621	Hide	1162
				Body	37			Body	345
				Total	150			Total	1507

It will be seen that the rats on the stock diet and those getting colloidal sulfur remained at approximately the same weight throughout the experiment, and that the cystine contents of these groups (both bodies and hides) were nearly equal at its termination. The rats receiving the cystine supplements increased both in weight and cystine content, the latter increase appearing primarily in the hides. Although the increment in growth of the cystine control rats was only about half that observed by other workers, it was nevertheless definite and consistent. The fairly large amount of oil taken care of by the rats may be responsible for these smaller increases in weight.

Conclusion. Under the conditions of this experiment colloidal sulfur was not utilized by rats either for production of cystine or for growth.

[3] Virtue, R. W., and Lewis, H. B., *J. Biol. Chem.*, 1934, **104**, 415.

9071

Heart and Skeletal Muscle During Recovery from Hyperthyroidism.*

M. Bodansky and J. F. Pilcher. (With the technical assistance of Virginia B. Duff.)

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The object of these experiments was to determine the correlation, if any, between the reparative changes in muscle during convalescence from the hyperthyroid state and the return of the creatine concentration to normal levels.

Rats were made thyrotoxic by the daily administration of 1 mg. of synthetic thyroxine (Hoffmann-La Roche) for 23 days. At this stage a number of the animals were sacrificed and heart and skeletal muscle removed and subjected to chemical analysis and microscopic study. From the remaining rats muscle was obtained from the left thigh for similar purposes. These animals were then allowed to recover for varying periods and sacrificed. Chemical analysis and histologic study of the corresponding muscles of the right thigh made it possible to determine such improvement as may have occurred. The regeneration of the myocardium was evaluated by comparison with controls and on the basis of considerable other data previously reported from this laboratory.

The changes produced in muscle in hyperthyroidism include swelling of the fibers and vacuolization. The cross striations become dim, or disappear; the fibers lose their individuality and undergo atrophy, hyalinization and fibrosis. Reduction in the concentration of glycogen and creatine accompany these changes. In rats maintained in a fair state of nutrition and at a suitable environmental temperature, regenerative changes, such as proliferation of the sarcolemma may go hand in hand with the degenerative process, but as may be anticipated, the former become more prominent when thyroxine is withdrawn. Gradually the cross-striations both in the skeletal and cardiac musculature become more visible and the fibers more discrete. However, in our experiments, even after 28 days, the reparative process was not complete, signs of previous and existing damage still remaining.

In Figure 1 are represented the values for creatine obtained in a typical series of experiments. On the average, the preliminary

* Aided by a grant from the National Research Council.

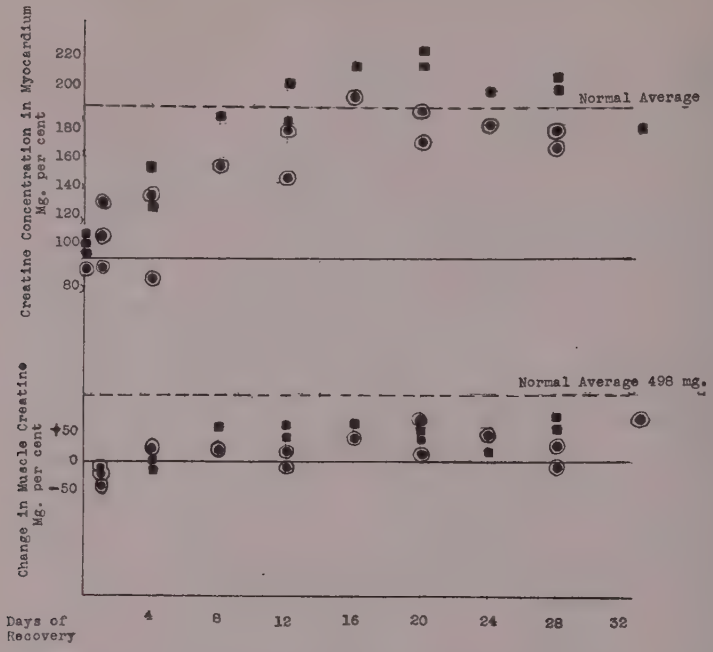

FIG. 1.

The return of the creatine concentration of the heart to normal during recovery from hyperthyroidism is illustrated in the upper part of the chart. The solid line in the lower portion represents the creatine concentration of the biopsy specimen, while the circles and squares above and below the line represent the change in concentration after partial recovery. Male rats are designated by circles; females by squares.

treatment with thyroxine produced about 20% reduction of creatine in skeletal muscle and 40-50% in the myocardium. Much further lowering of heart creatine seldom occurs owing to the intervention of death from heart failure. From the results it appears that the creatine concentration of the heart tends to return to normal rapidly, restitution being well in advance of tissue repair. In skeletal muscle, on the other hand, the earlier regenerative changes, especially in the presence of an inflammatory process, are often unaccompanied by any change in creatine. Later the increase in creatine concentration more or less parallels anatomic improvement.

Conclusions. In the hearts of rats recovering from hyperthyroidism restoration of the creatine concentration occurs in advance of any marked anatomic improvement and it is therefore not inconsistent to find normal values in the presence of moderate myocardial damage. Reparative processes occur somewhat more gradually in skeletal than in cardiac muscle and the restoration of creatine to normal more nearly parallels the degree of tissue regeneration.

9072 P

pH Changes of Muscle During and After Contraction.

M. Dubuisson. (Introduced by L. Michaelis.)

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Since Meyerhof and Lipmann,[1] and Meyerhof, Moehle and Schulz[2] studied the exchange of CO_2 in frogs' muscle during fatigue, we know that under anaerobic conditions the pH of the muscle, when subjected to periodic stimulations, will first increase and then gradually decrease. However, the method applied by these authors does not show the pH changes during the activity itself, *i. e.*, contraction and relaxation of the muscle. Margaria and Pulcher[3] attempted to show the pH change during contraction itself by coloring the muscle with bromocresol purple. They found a change in color during the contraction corresponding to an increase in pH of about 0.5. According to Margaria,[4] however, the same changes occur when the muscle is mechanically stretched. Later Margaria and von Muralt[5] described a photo-electric method with which it would be possible to record those changes of color. On summarizing, as far as I know, no records showing the pH changes during and after contraction have been published. I succeeded in obtaining such records during tetanus by an entirely different method. Its principle is to cover a part of the surface of the muscle with a glass electrode and to record the pH changes occurring on stimulation.

The glass electrode consists of a membrane of suitable glass sealed to the end of a tube of ordinary glass. This is the first of the two types of glass electrode described by MacInnes.[6] The glass membrane is brought into contact with the wet external surface of the muscle. Glass electrode, muscle, and a potentiometer are mounted in series in the grid circuit of a Pliotron tube F.P. 54.[7] A galvanometer is placed in the plate circuit. The deflections of the galvanometer light spot due to a change in the grid voltage are recorded on a silver-bromide paper camera. The inside of the glass electrode tube contains 0.1 N HCl and a silver silver-chloride electrode.[8] The

1 Lipmann, F., and Meyerhof, O., *Biochem. Z.*, 1930, **227**, 84.
2 Meyerhof, O., Möhle, W., and Schulz, W., *Biochem. Z.*, 1932, **246**, 285.
3 Margaria, R., and Pulcher, C., *Boll. soc. Biol. sper.*, 1934, **9**, 879.
4 Margaria, R., *J. Physiol.*, 1934, **82**, 496.
5 Margaria, R., and v. Muralt, A., *Naturwiss.*, 1934, **22**, 634.
6 MacInnes, D. A., and Belcher, D., *J. Am. Chem. Soc.*, 1931, **53**, 3375.
7 General Electric Co.
8 Brown, A. S., *J. Amer. Chem. Soc.*, 1934, **56**, 646.

other half cell consists of a silver silver-chloride electrode immersed in Ringer solution, which, by means of a cotton thread, is held in contact with the tendon of the muscle. The muscle is kept in a glass chamber and, as a preparatory step for the experiment, is first immersed in a buffered Ringer solution for one hour through which is bubbled steadily a gas mixture of 95% O_2+5% CO_2. Then the Ringer solution is discarded and the chamber now bubbled with a mixture of 95% N_2+5% CO_2. The glass electrode is now put in contact with the muscle in the moist chamber and the muscle is stimulated through its nerve with the aid of an induction coil. Figure 1 shows a typical record.

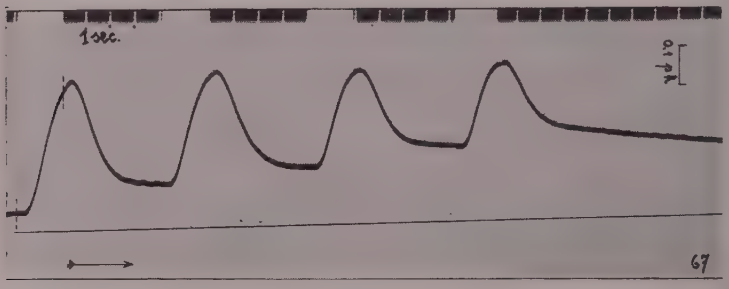

Fig. 1.

pH changes (increase up, decrease down) during and after four tetani, each of about 2 seconds, in the fresh gastrocnemius of a frog. Temperature: 25°C. At the starting point the pH was 6.91 (December 4, 1936). The small gaps in the time scale indicate seconds; the larger gaps show the periods of stimulation.

One sees that half a second after the beginning of the stimulation the thin liquid film outside the muscle, which is in contact with the glass membrane, begins to become more alkaline, gradually becoming more and more so. The maximum change is reached about half a second after the end of a stimulation and amounts to about 0.3 pH unit. After this maximum has been reached the change reverses and the film becomes gradually more and more acid. Other records have shown that one minute after a 2-second tetanus the pH is lower than it was in the beginning. The records differ according to the state of fatigue. In a nearly exhausted muscle the increase of pH during contraction is very much less, on the other hand, the decrease of pH during recovery is greater. On varying size, shape and thickness of the glass membrane it could be clearly seen that these factors have no influence on the type of the records. On the other hand it is very important to control carefully certain technical factors concerned with the exchange of CO_2. The method

is based on the fact that CO_2 freely passes across the membrane of the muscle, but the other substances involved in buffering effects are exchanged either much more slowly or not at all. For this reason this method shows no pH change at all on stimulation when the muscle has been previously freed as much as possible from CO_2 by bubbling with pure nitrogen. Furthermore, it is necessary to isolate that part of the muscle surface which is in contact with the glass membrane, from the gas mixture in the chamber. This can be easily achieved by a rubber ring. So the muscle is first brought into gas equilibrium with the gas mixture bubbling through the chamber, then the glass membrane and the rubber wall surrounding it are brought into contact with the muscle, cutting off the gas exchange between the muscle surface underneath the glass membrane and the outside gas atmosphere. Under these conditions any change in pH inside the muscle is followed, owing to the CO_2 exchange, by a pH change in the film of moisture which is situated between the glass electrode and the muscle. It is the pH change in this film of moisture due to the CO_2 exchange that we are recording with this method.

On summarizing, this method permits of the study, without any damage to the muscle fibers, of the surprisingly fast exchange of CO_2 which results from chemical processes occurring during and after contraction, between the inside of the muscle and the moist film covering its surface. The rapidity of these exchanges permits the recording of the changes of pH with relatively little lag of time (half a second). A full description of the methods and results will soon be published.

I wish to express my sincere thanks for the support given to this work by Dr. L. Michaelis, to whom I am indebted for many helpful suggestions and advice.

This work was done during a tenure of a fellowship granted by the C.R.B Educational Foundation, whose generous assistance I wish to acknowledge.

9073 P

A Complement-Fixation Reaction Involving the Rabbit Papilloma Virus (Shope).

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The rabbit papilloma virus[1] is a notably stable and specific pathogenic agent which lends itself readily to quantitative immunological work.[2] The present paper reports a complement-fixation reaction with extracts of the papillomas caused by this virus and sera effective against it.

The technic used was essentially that described for viruses by Bedson and Bland[3]; it is based largely on the principles established by Bordet,[4] Dean,[5] and Merrill.[6] To 0.2 cc. of the appropriate dilution of inactivated serum was added 0.2 cc. of saline containing 2 units of guinea pig complement (titrated immediately beforehand), and then 0.2 cc. of the proper dilution of antigen. The antigen consisted of a centrifugalized, or filtered, saline suspension of glycerolated, or frozen and dried, papilloma tissue which had been ground with sand in a mortar and extracted overnight (or longer) in the refrigerator. The antigen was heated at 56°C. for 30 minutes immediately before use. Occasionally antigen was put into the tubes first and serum third, but complement was always added second. These reagents were then gently shaken and allowed to stand 2 hours at room temperature. 0.4 cc. of a suspension consisting of 0.2 cc. of 5% sheep cells mixed 10 minutes beforehand with 2 units of amboceptor in 0.2 cc. saline was then added to the tubes, which were again shaken and incubated at 37°C. for 30 minutes. Readings in terms of fixation were made immediately after the incubation and again after the tubes had been in the refrigerator overnight.

Serum Tests. The sera of 15 domestic and 5 wild cottontail rabbits bearing papillomas caused by the Shope virus were found to bind complement in the presence of antigen, whereas the sera of 6 normal domestic and 3 normal cottontail rabbits gave no fixation when tested concurrently. In further experiments the sera of 6

1 Shope, R. E., *J. Exp. Med.*, 1933, **58**, 607.

2 Kidd, John G., Beard, J. W., and Rous, Peyton, *J. Exp. Med.*, 1936, **64**, 63, 79.

3 Bedson, S. P., and Bland, J. O. W., *Brit. J. Exp. Path.*, 1929, **10**, 393.

4 Bordet, J., and Gengou, O., *Ann. Inst. Pasteur*, 1901, **15**, 289.

5 Dean, H. R., *Z. Immunitätsforsch.*, 1912, **13**, 84.

6 Merrill, M. H., *J. Immunol.*, 1936, **30**, 169.

domestic rabbits bearing papillomas induced by tar, as well as specimens from 10 domestic rabbits resistant to the fibroma or myxoma virus,* have failed to fix complement on test with known complement-binding antigens.

Comparative tests were made of the virus-neutralizing and complement-fixing properties of the sera of 6 domestic rabbits bearing Shope papillomas and these properties were found to vary together. Two of the sera showed marked capacity to bind complement, and these neutralized the virus most effectively; 2 had little power to bind complement or to neutralize the virus, and the remaining 2 proved intermediate as regards both titers.

Tests with Extracts of Various Papillomas. Complement-binding antigens have been procured from the glycerolated Shope papillomas of wild cottontail rabbits, jack rabbits, snowshoe hares, and domestic rabbits. These differed notably in effectiveness, however, the complement-binding power of an extract being roughly proportional to its infective titer. Non-infective extracts from the papillomas of a number of domestic rabbits have failed to bind complement when tested in various dilutions with strong antisera. Zone phenomena have sometimes been observed with inhibition of fixation when the proportion of antigen to antibody has been too great.†

Heating 2 antigenic filtrates for 30 minutes at 60°C. did not notably affect their complement-binding power, but this was greatly diminished or destroyed by heating at 63°, 66°, or 69°C. Comparative tests indicate that the infective Shope virus contained in these filtrates was likewise unaffected at 60°C. but was largely or completely destroyed by heating for 30 minutes at 63°, 66°, or 69°C.

The following experiment provided further indications of the existence of a relation between the complement-binding and infective capacities of papilloma extracts. Five per cent extracts of glycerolated papillomas from 2 cottontail rabbits were kept 7 and 21 days respectively in the refrigerator, after which 5 cc. samples of each were put through filters of widely different pore size. The filtrates (1.5-2.0 cc. in total amount) were compared for complement-binding power and for ability to produce papillomas on the skin of susceptible rabbits. The Berkefeld V filtrates fixed complement completely, while the Berkefeld W filtrates bound it only slightly if at all when tested with the same antiserum, and the extracts filtered through single Seitz E K discs were without perceptible

* These fibroma-myxoma antisera were generously given by Dr. R. E. Shope, and the amboceptor by Dr. Kenneth Goodner.

† In a collateral test done recently with a potent complement-binding antigen and undiluted antiserum, visible precipitation occurred.

power to fix complement. Inoculations showed that the infective virus had passed through the Berkefeld V candles in large amount, whereas little had come through the Berkefeld W and Seitz filters.

Summary. A complement-fixation reaction is described, with extracts or filtrates of papillomas containing infective Shope virus and antisera effective against the latter. The implications of the work are being studied.

9074 P

Intradermal Venom Reaction. A New Method of Determining Capillary Fragility.

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The intradermal injection of a potent moccasin snake venom in sufficient concentration into animals or human beings is followed by a local ecchymosis. However, dilute solutions, such as 0.1 cc. or 0.2 cc. of 1 :3000 will not normally be followed by ecchymosis.[1]

The dry scales of moccasin venom are dissolved in physiologic saline with merthiolate added as a preservative. The titration of the venom is carried out by the use of the chicken embryo.[2] The lowest dilution of venom in 1 cc. amounts that produces hemorrhage of the vascular tree in a four-day-old chicken embryo in a 3-hour period is considered to contain 10 hemorrhagin units. This is usually a 1 :3000 solution. Thus 0.1 cc. of standardized venom contains one hemorrhagin unit. Such standardized venom solution will not show deterioration in 4 months at icebox temperature.

The intradermal venom test consists of injecting 0.1 cc.-0.2 cc. (1 or 2 hemorrhagin units) of standardized moccasin venom intradermally and reading the reaction within 30 minutes to one hour, 0.1 cc.-0.2 cc. of physiologic saline injected at the same time serving as a control. A positive test is one in which there is a definite capillary rupture or ecchymosis at the injection site within an hour. The ecchymosis may be one centimeter or more in diameter. A delayed positive reaction is one that shows a diffuse ecchymosis in 12

1 Peck, Samuel M., *Arch. Derm. and Syph.*, 1933, **27**, 312.

2 Witebsky, C., Peck, S. M., and Neter, E., PROC. SOC. EXP. BIOL. AND MED. 1935, **32**, 722.

to 24 hours. A negative test is one that shows no hemorrhage. Confusing reactions such as edema and erythema of various degrees can be differentiated after a little experience.

Because a small area of skin is involved in the venom reaction, it can be repeated at frequent intervals and it can be used to determine local capillary fragility within a very limited area. Furthermore, capillary vulnerability to the hemorrhagins may be measured in areas inaccessible to the tourniquet test, such as the cheek, mucous membranes, gums and center and periphery of skin lesions.

It was shown[3] that the most important information thus far obtained by the test was in thrombocytopenic purpura hemorrhagica, in the classification of such cases and in their prognosis. The persistence of a positive reaction to successive tests or a reversal to a negative reaction was of value in determining the trend of the purpuric state. The test could also be used as a guide for venom therapy in the management of cases with chronic thrombocytopenic purpura hemorrhagica, and it is an important indication for splenectomy. After the splenectomy it accurately predicted which of the cases would benefit by the operation.

Since the venom reaction is a test for the purpuric state, it was positive in symptomatic purpura associated with benzol poisoning, aplastic anemia, leukemia, subacute endocarditis, malignancies, nephritis, etc. Although the purpura in those cases was closely related to the platelets, the test did not necessarily serve as an indicator of the platelet count alone.

The intradermal venom test was negative in the affected areas (usually the face) as well as in other parts of the body in a number of cases of chronic discoid lupus erythematoides. In some of the acute forms of this disease the test was negative even when performed in the skin of the cheek showing the lesion. However, a strongly positive test was obtained in 3 cases of lupus erythematoides disseminatus of the acute fatal variety. Such differences in reaction might prove of importance in differentiating the various types of this disease.[4]

The venom test gave varying reactions in different types of purpura not associated with a diminution of the platelets and proved of some use in differential diagnosis. In orthostatic purpura of the legs the venom test was positive only in the affected areas. In toxic purpura where the legs were mainly affected, the test was positive

[3] Peck, S. M., Rosenthal, N., and Erf, L., *J. A. M. A.*, 1936, **106**, 1783.

[4] Baehr, G., Klemperer, F., and Schifrin, A., *Trans. Assn. Am. Physicians*, 1935, **1**, 139.

not only in that area but on the arms as well. Thus the test differentiated a local from a general predisposing factor as the cause of the purpuric manifestations. Three cases of Schonlein's Henoch purpura gave a negative test. However, a positive reaction was obtained in all regions of the body tested in a patient in whom a similar diagnosis was made. The first 3 cases were probably observed during an inactive stage of the disease. In this way the test serves as an indicator of an active capillary toxic factor.

In 14 cases of coronary sclerosis and in 3 cases of coronary thrombosis a positive test of a peculiar type was observed. Instead of the usual bluish discoloration appearing in one hour, there developed at the injection site a brownish area about one centimeter in diameter. This has not been seen in any other condition.

A negative reaction was obtained in hemophilia.

The character of telangiectatic and vascular lesions could also be studied by means of the test. Vascular lesions of nevoid type such as flat hemangiomas gave a negative test when the injection was made at the margin of the affected area. Obviously we were dealing with normal blood vessels. On the other hand, injections into the skin in the vicinity of a lesion of angioma serpiginosum gave a strongly positive venom test, suggesting the presence of altered capillary structure.

Summary. A new capillary fragility test based on intradermal injections of titrated moccasin snake venom is described. This test was found helpful in the management of thrombocytopenic purpura hemorrhagica. Its applicability as a guide to local, general and toxic capillary changes is discussed.

9075 P

Subneural Gland of Ascidian *(Polycarpa tecta)*: an Ovarian Stimulating Action in Immature Mice.

B. M. HOGG. (Introduced by H. B. Williams.)

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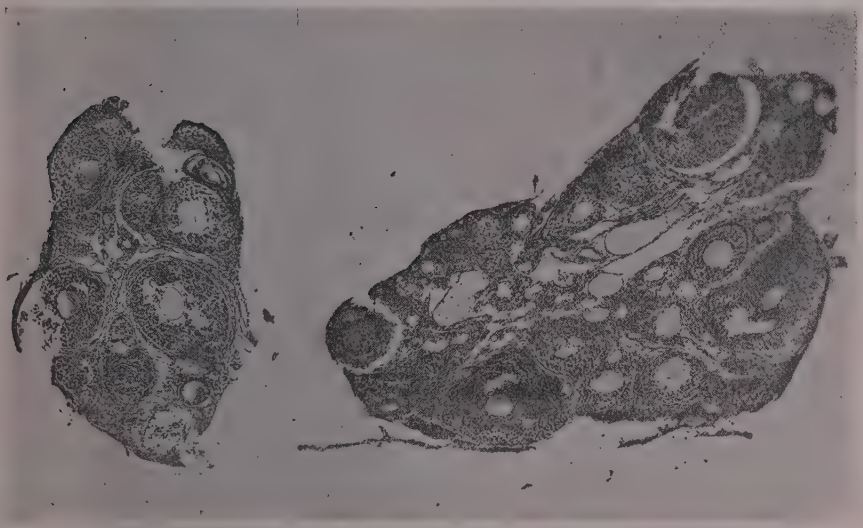
The subneural gland of the ascidian has long been the subject of controversy in regard to its probable function. On developmental grounds it has been denied admission to phylogenetic series dealing with the pituitary and until quite recently there has been no physiological evidence which might justify including it in such a series on a functional basis.

Butcher[1] demonstrated the presence of the oxytocic principle in the gland of Molgula and this result suggested the possibility of obtaining other pituitary effects such as the ovarian stimulating action in immature animals. The present work deals with an attempt to demonstrate such an effect using the gland of the large solitary ascidian *Polycarpa tecta* common in Bermuda waters.

In 1934, through the courtesy of the Bermuda Biological Station for Research, 70 fresh specimens of *Polycarpa tecta* were obtained and the ganglion, subneural gland and dorsal tubercle removed and desiccated in acetone. The dry weight was found to be 45 mg. and from a study of serial sections it was apparent that not more than 20-30 mg. of this weight could be considered glandular substance. Assuming a potency equal to that of mammalian whole gland it was not thought advisable to divide the material among more than 3 test animals. Accordingly, after pulverization and suspension in sterile isotonic saline equal amounts were injected subcutaneously into 3 nineteen-day-old mice. Three control animals received subcutaneous saline. None of the animals died and all were killed and autopsied on the fifth day.

Grossly the ovaries of the test animals were larger and appeared



"A" "B"

FIG. 1.
Test and control ovaries × 70. "A" control, "B" test.

[1] Butcher, Earl O., *J. Exp. Zool.*, 1930, **57**, 1.

more vascular. The collective weights to the nearest milligram compared as follows: Test, 25 mg.; control, 8 mg.

Microscopic examination of the ovaries showed an increase in vascularity and in the number and size of the follicles in the test specimens. (Fig. 1.)

The number of test animals is small and the response obtained is not striking but the results do seem to suggest further evidence in favor of a pituitary function for the subneural gland.

9076 P

Relation Between Human Vaginal Smears and Body Temperatures.*

B. B. RUBENSTEIN† AND D. B. LINDSLEY. (Introduced by T. Wingate Todd.)

From the Brush Foundation, Western Reserve University.

The inaccessibility of ovaries and uterus no longer handicaps the study of ovarian function in the human female since Papanicolaou and his associates[1] established the diagnostic value of vaginal smears. The smear technique, however, is necessarily surrounded with such precautions as to make it too elaborate for general use. In our effort to find a simpler but nevertheless reliable substitute we have correlated body temperature with changes in the vaginal smears on the ground that body temperature is known to vary in a regular manner during the menstrual cycle.[2]

The cycle of vaginal smears can be conveniently divided into 6 phases: (1) menstrual (3-7 day), (2) post-menstrual (3-6 day), (3) preovulative (1-5 day), (4) ovulative (1-3 day), (5) post-ovulative (5-8 day), (6) premenstrual (3-7 day). Rectal temperatures, taken before rising in the morning, *i. e.*, between 6 A. M. and 7 A. M., and vaginal smears were obtained daily during 5 complete menstrual periods of 4 young adult women without pelvic abnormality.

Table I summarizes the data. The lowest temperatures occur in the ovulative phase, the highest in the premenstrual phase. The

* Fellow of the General Education Board.

† Supported by a grant from the General Education Board.

1 Papanicolaou, G. N., *Am. J. Anat.*, 1933, **52**, 519.

2 Seward, G. H., *Psych. Bull.*, 1934, **31**, 153.

TABLE I.
Average Temperature Changes During 5 Menstrual Cycles in Each of Four Subjects.

		Menstrual	Post-mens.	Pre-ovul.	Ovulative	Post-ovul.	Premenstr.	Correlation coefficient
Subj. I	Temp.	97.5	97.4	97.4	97.1	97.8	97.9	+.850
	S.D.*	.40	.29	.36	.22	.41	.38	
Subj. II	Temp.	98.4	98.5	98.2	98.1	98.6	98.9	.860
	S.D.	.23	.31	.30	.16	.33	.27	
Subj. III	Temp.	97.9	97.7	97.6	97.6	98.5	98.4	.833
	S.D.	.31	.24	.22	.24	.39	.45	
Subj. IV	Temp.	98.3	98.3	98.1	97.8	98.4	98.7	.912
	S.D.	.28	.18	.27	.23	.30	.21	

*Standard deviation.

rank-correlation coefficients of body temperatures with vaginal smears arranged in the order (1) ovulative, (2) preovulative, (3) post-menstrual, (4) menstrual, (5) post-ovulative, (6) premenstrual are statistically significant, the values ranging from +0.833 to 0.912.

9077

Effect of Massive Doses of Adrenal Cortical Hormone on the Albino Rat.*

JESSIE L. KING. (Introduced by G. A. Harrop.)

From the Department of Physiology and Hygiene, Goucher College, Baltimore, Md.

The effect on the reproductive system, whether direct or indirect, of a deficiency in the adrenal-cortical hormone is well established. A hypersecretion has been held responsible for adrenal virilism but attempts to produce sexual precocity or other abnormalities of the reproductive system by injecting the hormone into normal animals have not given uniform results.

Howard and Grollman,[1] in the latest investigation on the effect of hypersecretion, injected a moderate excess of cortical hormone into normal rats for a maximum period of 57 days. Their results were negative and they attribute the positive results reported by some others to nonspecific impurities in their extracts.

The amount of hormone which could be injected in any of these experiments, however, is not comparable to the excess which might be secreted by a cortical tumor. Fortunately the new method of

* The investigation was carried out with the aid of a grant from the Committee on Scientific Research of the American Medical Association.

[1] Howard, E., and Grollman, A., *Am. J. Physiol.*, 1934, **107**, 480.

Grollman, Firor, and Grollman[2] of absorbing the adrenal-cortical hormone on charcoal, makes it possible to give larger quantities *ad libitum* of a potent extract than could possibly be injected. It also has the advantage of making the hormone continuously available and the conditions, therefore, are more comparable to those of normal secretion. It seemed worthwhile to reëxamine the problem and to test the effect of excessive amounts of the hormone, especially on growth and on the reproductive system of normal rats.

Five female albino rats of Wistar stock with their littermate controls were used. The group was kept small because of the expense of the extract. The experiment was started when the rats were 26 days old and was continued for 26 days for one and from 72 to 89 days for the other 4, in order to give time for all possible changes to express themselves. The cortical-hormone combination was kindly furnished by Dr. George A. Harrop. It was prepared in his laboratory according to the method of Grollman, Firor, and Grollman.[2] It was assayed on adrenalectomized rats and its potency assured. One gram was equivalent to 200 or 300 gm. of fresh beef cortex. This charcoal-adsorbate was mixed in gradually increasing amounts with a weighed standard ration until the dose for each rat reached a gram per day. Those fed for long periods received then the equivalent of from 15,000 to 18,000 gm. of fresh cortex, a much greater quantity than has been reported in any previous work.

Normal sexual development was indicated by the opening of the vagina which averaged for both groups 39 days, by the regularity of the cycles, as indicated by daily vaginal smears and by the weights of the ovaries at autopsy and their histological picture compared

TABLE I.

Summary of Data on the Effect of Massive Doses of Adrenal-Cortical Hormone on Long Period Experiments on 4 Albino Rats. All Numbers Are Averages.

	Experimental	Control
Initial age in days	26	26
Duration of experiment	79.2	79.7
Initial body weight	54.7	54.2
Final body weight	222.0	212.0
Age at opening of vagina	39.0	39.4
Weight of ovaries in mg.	47.03	43.50
Ovaries, % of final body wt.	0.0211	0.0205
Weight of adrenals in mg.	28.2	28.0
Adrenals, % of body wt.	0.0127	0.0132
Weight of thyroid	16.42	20.75
Thyroid, % of body wt.	0.0739	0.0978
Total gm. charcoal-combination in equivalent gms. cortex	18650	
Gm. charcoal-combination fed per rat per day, in equivalent gm. cortex	234	

2 Grollman, A., Firor, M., and Grollman, E., *J. Biol. Chem.*, 1935, **110**, 189.

with those of the controls. Other endocrine glands were studied and were similar in their weights and in histological structure to those of the controls. Although the experimental animals gained a little faster than the others, at autopsy, the average difference was too small to be regarded as significant. A summary of these data is given in Table I.

Conclusion. The negative results obtained from the feeding of massive doses of charcoal adsorbate, prepared from beef adrenal cortex may indicate that the substance in the adrenal cortex, which stimulates the reproductive system, is a different hormone from that demonstrated by Harrop[3] as affecting the metabolism of salt and water and it may not be extracted by the methods at present in use for extracting the latter. The excellent condition of the experimental animals is evidence that the substance is not toxic.

9078 P

Effect of Stimulus of Suckling Upon Galactin Content of the Rat Pituitary.

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In a previous experiment we were able to demonstrate that the stimulus of suckling and/or the removal of milk from the mammary glands markedly decreased the galactin content of the post-partum rat pituitary gland.

Selye *et al.*[1] have shown that the act of suckling prevented the involution of the mammary glands of the rat during a period of 14 days, even though the milk ducts were ligated. Turner and Reineke,[2] working with 2 goats, suspended milking on one side of the udder about 30 days after parturition, while the other side was milked regularly. Histological sections of biopsy specimens taken 65 days after the beginning of the experiment revealed that the alveolar structure was still well defined on the unmilked side. The procedure

[3] Harrop, G. A., *Johns Hopkins Hosp. Bull.*, 1936, **59**, 11 and 25.

* Contribution from the Department of Dairy Husbandry, Missouri Agricultural Experiment Station, Journal Series No. 493.

[1] Selye, H., Collip, J. B., and Thomson, D. L., *Endocrin.*, 1934, **18**, 237.

[2] Turner, C. W., and Reineke, E. P., *Mo. Agr. Exp. Sta. Res. Bul.*, 1936, 235.

employed by Selye in his work gave us a technique whereby we could determine if the decrease in the galactin content of the rat pituitary gland under the influence of suckling was due to the stimulus of suckling or to the removal of milk from the mammary glands.

In 11 rats, 36 hours postpartum, the litters were removed and the primary milk duct of each mammary gland was ligated, making it impossible to remove milk from the mammary glands. Twelve hours later the litters were returned to their mothers and the young allowed to nurse for 3 hours. The mothers were then sacrificed, their pituitaries removed and weighed. The pituitaries were then assayed by injecting them intradermally over the crop gland of the common pigeon. The results are summarized in Table I.

TABLE I.
Effect of Stimulus of Suckling upon Galactin Content of the Rat Pituitary.

Rat No.	Body Wt., gm.	Total Pituitary Wt., mg.	Bird Units per Pituitary Gland	Bird Units per mg. Pituitary Tissue	Bird Units per 100 gm. Body Wt.
58	253	9.7	6.50	.67	2.57
57	241	12.6	5.87	.47	2.44
53	215	13.6	6.12	.45	2.85
56	192	10.9	3.67	.34	1.91
51	182	9.5	5.00	.54	2.75
60	180	9.1	4.75	.52	2.64
62	175	9.9	6.00	.61	3.43
63	170	7.6	5.00	.66	2.94
61	160	7.5	5.12	.68	3.20
54	151	9.7	3.67	.38	2.43
52	150	6.7	5.50	.82	3.67
Average	188	9.7	5.20	.56	2.80

Pituitary glands from rats in which the litters were removed 36 hours after parturition and then sacrificed 15 hours later contained, on the average, 9.2 bird units per pituitary gland, as compared with 5.2 bird units per pituitary gland from rats in which the young nursed but secured no milk. This value of 5.20 bird units per pituitary gland is somewhat higher than the value, 3.06 bird units per pituitary gland observed in rats in which the young were allowed to suckle and remove the milk.

Conclusion. The stimulus of suckling definitely decreases the galactin content of the rat pituitary gland even when no milk is removed.

9079

IV. p-Bromophenylmercapturic Acid and Ethereal Sulfates Synthesis in Dogs Maintained on Diets of Varying Sulfur Content.

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Hele[1] and White and Lewis[2] have suggested that when the dietary sulfur is too low to meet the needs for detoxication of bromobenzene to yield mercapturic acid, the dog is forced to employ to a larger extent other paths of detoxication of bromobenzene, namely, oxidation to bromophenol and conjugation with sulfuric acid. This conclusion was based on the observation that following the feeding of bromobenzene to dogs maintained on diets of various sulfur contents, the neutral sulfur of the urine was most pronounced on a high-sulfur diet, less so on a low-sulfur diet. On a low-sulfur diet, coincident with a lower output of the neutral sulfur, higher values for the ethereal sulfates in the urine were observed. In view of the limitations of the significance of the fluctuations of the neutral sulfur in the urine following the administration of bromobenzene,[3] it seemed of interest to check the suggestions of Hele[1] and White and Lewis,[2] mentioned above, using a recently developed direct method for the estimation of mercapturic acid in the urine.[4]

Each of 4 female dogs were kept in individual metabolism cages and fed each of the diets described in Table I. After the completion of the experiments on the casein diet, each dog was then fed the low-

TABLE I.
Composition of Diets.*

	Casein	Low-protein	Protein-free
Casein, gm.	43.7	22.0	
Sucrose, gm.	40.6	62.3	84.3
Vitavose, gm.	11.6	11.6	11.6
Salt mixture,† gm.	1.4	1.4	1.4
Bone ash, gm.	2.7	2.7	2.7
Butter fat, gm.	7.0	7.0	7.0
Lard, gm.	17.0	17.0	17.0
Nitrogen, %	4.92	2.57	0.20
Sulfur, %	0.250	0.124	0.030

*The diets were devised according to: Cowgill, G. R., Deuel, H. J., Jr., and Smith, H. H., *Am. J. Physiol.*, 1925, **73**, 106.

†As used by Karr, W. G., *J. Biol. Chem.*, 1920, **44**, 255.

1 Hele, T. S., *Biochem. J.*, 1924, **18**, 586; 1926, **20**, 606.

2 White, A., and Lewis, H. B., *J. Biol. Chem.*, 1932, **98**, 607.

3 Stekol, J. A., *J. Biol. Chem.*, 1937, **117**, 147.

4 Stekol, J. A., *J. Biol. Chem.*, 1936, **113**, 279.

protein diet, and similar experiments carried out. After the low-protein diet, the dogs were fed the casein diet again until they recovered from the protein starvation while receiving the low-protein diet. Nitrogen and sulfur balance was taken as a criterion of such recovery. Then they were fed the protein-free diet. In all cases, our dogs received food yielding 60-65 calories per kilo of body weight, each dog receiving the same amount of food irrespective of the nature of the diet. The urine was collected every 24 hours by catheterization and the food was fed immediately after, at 9 a. m. One gm. bromobenzene was fed in a gelatine capsule daily for 4 consecutive days while feeding each of the 3 diets. The urine was analyzed by methods described previously[5] p-bromophenylmercapturic acid was determined by the recently described method,[4] until the urine collected for several days showed the absence of the acid. Water was allowed *ad libitum*. The ethereal sulfate sulfur values, as presented in Table II, indicate the rise in the output of ethereal sulfate sulfur on the days of administration of bromobenzene. The values were computed by deducting from the figures obtained on the day of feeding bromobenzene, the value for ethereal sulfates secured on the preceding days, usually the average of 3 to 4 days. The ethereal sulfate sulfur values were quite uniform,

TABLE II.
Excretion of Mercapturic Acid and Ethereal Sulfates on Diets of Varying Sulfur Content.

Diet	Casein		Low-protein		Protein-free	
	N	S	N	S	N	S
Intake	gm. 4.92	gm. 0.250	gm. 2.57	gm. 0.124	gm. 0.20	gm. 0.030
	Urinary Output					
Day	Mercapturic acid mg.	Ethereal SO_4S mg.	Mercapturic acid mg.	Ethereal SO_4S mg.	Mercapturic acid mg.	Ethereal SO_4S mg.
1*	560	26	568	30	600	27
2*	732	27	575	36	613	40
3*	823	30	580	40	405	41
4*	921	29	643	43	376	50
5	175	2	286	10	286	21
6	125		100		143	
Total	3336	114	2752	159	2423	179
% Bromobenzene detoxicated	38.0	14.2	31.3	20.0	27.6	22.4
Total % Bromobenzene detoxicated	52.2		51.3		50.0	

*1 gm. bromobenzene in a gelatin capsule fed at 9 a.m.

[5] Stekol, J. A., *J. Biol. Chem.*, 1934, **107**, 225; 1934, **107**, 641; 1935, **109**, 147.

varying from 18 to 20 mg. per day, and remained practically unaffected by the change of diet.

For the sake of economy of space, typical results are presented in a summarized form in Table II. The data indicate that on the casein diet, the repeated administration of bromobenzene was followed by an apparently progressive increase in the synthesis of mercapturic acid. This apparent increase we attribute to the accumulation of mercapturic acid in the body with each successive dose of bromobenzene, due to a lag in the excretion of mercapturic acid.[3] No significant increase in the ethereal sulfate synthesis was noted on repeated feeding of bromobenzene on the casein diet. On the low-protein and the protein-free diet, the extent of synthesis of mercapturic acid was lower, on the protein-free diet being the lowest. The largest amount of ethereal sulfates was formed on the protein-free diet. Comparison of the per cent detoxication of bromobenzene on the 3 diets shows that the total amount of bromobenzene taken care of by our animals to yield ethereal sulfates and mercapturic acid, irrespective of the dietary sulfur, was about the same, 50-52% of the dose fed. The nature of the detoxication, however, was quantitatively different: on a low-protein or protein-free diet, the animal is apparently forced to employ the mechanism of ethereal sulfate formation to a greater extent than on a casein diet. The results are in accord with the observations of Hele[1] and White and Lewis[2] concerning the existence of 2 independent mechanisms of detoxication of bromobenzene in dogs. It should be, however, emphasized again that these observations by no means demonstrate the direct dependence of the extent of the synthesis of p-bromophenylmercapturic acid in dogs on the cystine or methionine content of the diet. Our data show that even though our diets varied in their sulfur content from 0.248 to 0.020%, *on the first day of maintenance on these diets our dogs yielded nearly the same amounts of mercapturic acid and ethereal sulfates.* It is improbable that cystine and methionine of the diet remained in circulation 24 hours after the last administration of food. The available data indicate that l-cystine and dl- or l-methionine, when fed to dogs in amounts as present in our casein diet, are readily oxidized to yield inorganic sulfates.[6] Had the extent of the synthesis of mercapturic acid been dependent directly on the dietary sulfur, no such constancy in the output of mercapturic acid on the 3 diets could be expected. We suggested previously that the immediate source of sulfur of the mer-

[6] Pirie, N. W., *Biochem. J.*, 1932, **26**, 2041; Stekol, J. A., and Schmidt, C. L. A., *Univ. California Pub. Physiol.*, 1933, **8**, 31; Virtue, R., and Lewis, H. B., *J. Biol. Chem.*, 1934, **104**, 59.

capturic acid in all cases, irrespective of the dietary sulfur, is the tissue sulfur.[3] When l-cystine, cysteine, dl- or l-methionine were fed to dogs kept on the casein diet, no retention of the sulfur of these amino acids was observed[5]; nor was there any increase in the extent of the synthesis of p-bromophenylmercapturic acid as compared to that on the casein diet alone when l-cystine or dl-methionine were added to the casein diet.[3] When these amino acids were added to the low-protein diet, a retention of the sulfur of the amino acids was observed[5]; also an increased synthesis of p-bromophenylmercapturic acid as compared to that on the low-protein diet alone was noted.[3] It seems that when the dog is maintained on the casein diet, its sulfur stores, which are utilized in the detoxication of bromobenzene, cannot be further increased by the addition of l-cystine or dl-methionine to the diet, while on the low-protein diet supplemented by l-cystine or dl-methionine, a restoration of the tissue sulfur occurs and consequently an increased supply of the sulfur stores which are used in the detoxication of bromobenzene is available. As shown in Table II, on the first day of maintenance on the low-protein or the protein-free diets which were fed immediately after the casein diet, the stores of sulfur which the animal possessed while on the casein diet are still present and consequently the same amount of sulfur is available for the detoxication of bromobenzene on all 3 diets. On repeated administration of bromobenzene to dogs kept on a casein diet, these sulfur stores which are attacked by bromobenzene are restored at the expense of the dietary sulfur; while on the low-protein and the protein-free diets, less efficient recovery of the tissue and none at all respectively, takes place. Consequently, depletion of the sulfur stores of the animal follows, with subsequent increase in toxicity of bromobenzene with each new dose. Decreased synthesis of mercapturic acid is thus directly due to the depletion of the tissue sulfur available for the detoxication. It is of interest to mention in this connection that only l-cystine, cysteine and dl-methionine, but not taurine, were effective in augmenting the synthesis of p-bromophenylmercapturic acid in dogs.[2, 3] It will be remembered that only the first 3 amino acids are capable of replacing the tissue wear and promoting growth in animals kept on cystine-low diets. It seems that the ability of a sulfur-containing amino acid to restore the tissue sulfur of the animal is essential for its being an effective agent for augmenting the mercapturic acid synthesis in dogs.

Summary. 1. Dogs were fed diets of varying sulfur content and 1.0 gm. doses of bromobenzene were fed on 4 consecutive days on each of the diets. The synthesis of p-bromophenylmercapturic acid

and ethereal sulfates on these diets was estimated. 2. The extent of the synthesis of the mercapturic acid and of the ethereal sulfates is apparently a function of the nutritive state of the animal: when the animal was deprived of dietary sulfur, a decrease in the output of mercapturic acid and an increase in the output of ethereal sulfates was noted. 3. The results offer further support to the previous suggestion that the dietary sulfur is not the immediate source which is used for the detoxication of bromobenzene in dogs.

9080

Action of Parahydroxyphenylisopropylamine on Induced Cardiac Standstill.

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The pressor response of the experimental animal has been used almost exclusively in the investigation of the comparative pharmacological actions of the epinephrine-like compounds (the sympathomimetic amines).[1] In previous studies,[2] the cardiac standstill which may be induced in many individuals by pressure over the right carotid artery in the neck (carotid sinus reflex) was used as a basis for the investigation of the comparative activities of these substances on the human heart. In susceptible subjects a cardiac arrest of many seconds' duration can be induced consistently by compression of the carotid sinus. The standstill is the result of an active reflex stimulation of the vagus nerve so that the heart is temporarily deprived of its pacemaker, the sinus node.

Upon administration of drugs of the epinephrine series the cardiac standstill could be prevented due to the development of new centers of stimulus formation, usually in the ventricles. This effect on cardiac rhythmicity is definitely a sympathomimetic action as it could be produced by a number of sympathomimetic compounds and could not be affected by a variety of unrelated substances. The rate of the ectopic pacemaker was as an index of the intensity of the effect, and this served as a method for the comparative study of these substances using the human heart as the test object. As an illustration, after the intravenous administration of epinephrine 0.05 mg. the cardiac standstill was prevented by the development of a

1 Barger, G., and Dale, H. H., *J. Physiol.*, 1910, **41**, 19.

2 Nathanson, M. H., *Arch. Int. Med.*, 1934, **54**, 111.

ventricular rhythm with a rate of 60. A similar reaction was evoked by the intravenous injection of 100 mg. of ephedrine, giving an approximate ratio of ephedrine to epinephrine of 1:2000. Table I shows the comparative activities of a group of these substances as determined by this method.

TABLE I.

Comparative Activities of Sympathomimetic Amines on Cardiac Standstill.

Drug	Structural Formula	Approximate Ratio of Activity to l-epinephrine
l-epinephrine		1:1
d-epinephrine		1:20
[illegible]		1:10
Synthetic Substance		1:40
Synthetic Substance		1:40
Neosynephrin hydrochloride		1:100
Synephrin tartrate		1:400
Tyramine		1:1,200
Hordenine		1:6,000
Ephedrine		1:1,500 - 1:2,000
Phenylethanolamine		1:8,000
Catechol		Ineffective

The compounds were in the following forms: l-epinephrine, [illegible] amino 3,4 dihydroxy propylbenzene and the first-mentioned "Synthetic Substance" as hydrochlorides; d-epinephrine as the bitartrate; tyramine and hordenine, ephedrine and phenylethanolamine as the sulphates.

From a therapeutic standpoint there are clinical conditions in which it is highly desirable to have a substance available which possesses the property of preventing cardiac standstill and which is sufficiently stable to be active on oral administration and capable of producing a prolonged effect. Such a substance is especially ap-

plicable in the prevention of the ventricular standstill of heart block and the cardiac arrest which occurs in individuals with a hypersensitive carotid sinus reflex.[3] Ephedrine was the only substance available in the previous study which was effective when administered by mouth. The hydroxyamines as illustrated by the synephrine and tyramine were definitely more active but failed to show any effect on oral administration in large doses. Ephedrine possesses 2 features which limit its therapeutic value, (1) the comparative weakness of its action (ratio to epinephrine 1:2000 by this method) and (2) when administered in adequate dosage, unpleasant side effects due to stimulation of the central nervous system.

In chemical structure, parahydroxyphenylisopropylamine stands between ephedrine and epinephrine (Fig. 1). Alles[4] has shown

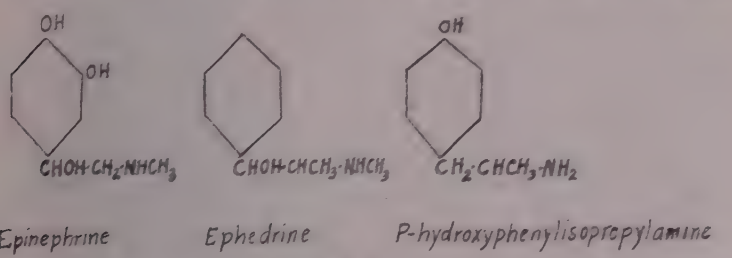

FIG. 1.

Chemical structure of epinephrine, ephedrine, and parahydroxyphenylisopropylamine.

that this substance has a more intense pressor activity than phenylisopropylamine, a compound closely related to ephedrine. In contrast to the hydroxy-amines previously studied, this substance is effective when administered by mouth. In a patient with a hypersensitive carotid sinus reflex, oral administration of the drug prevented the cardiac arrest.[5] In the present study, parahydroxyphenylisopropylamine hydrobromide* was administered to 8 individuals in whom a cardiac arrest could be consistently induced by pressure over the right carotid sinus. In each case the following procedure was carried out. A control electrocardiogram was made showing the induced cardiac arrest. A single dose of the drug was then administered by mouth which consisted in 6 instances of 60 mg. and in 2 cases of 40 mg. Electrocardiograms were then taken at intervals

[3] Weiss, S., and Baker, J. P., *Medicine*, 1933, **12**, 297.

[4] Alles, G. A., *J. Pharmacol. and Exp. Therap.*, 1933, **47**, 339.

[5] Prinzmetal, M., Personal communication.

* The parahydroxyphenylisopropylamine hydrobromide was supplied by the Smith, Kline and French Laboratories.

of 15 minutes and the pressure on the carotid sinus repeated. The cardiac standstill was abolished in every instance. In 5 individuals the standstill was eliminated due to the development of a new pacemaker in the ventricles, in 2 cases the sinus node retained its activity and in one instance beats from a supraventricular focus alternated with ectopic beats of ventricular origin. The effect was noted consistently within 30 minutes after the administration of the drug and the duration varied from one to 3 hours. In 3 subjects the experiment was repeated after an interval of several days to a week, using 100 mg. of ephedrine sulphate. In each instance ephedrine produced qualitatively the same effect as the parahydroxyphenylisopropylamine but the reaction was definitely less intense. The parahydroxyphenylisopropylamine produced a reaction which was earlier in onset and of longer duration. This is illustrated by Figs. 2 and 3. The qualitative similarity of the reaction is indicated in the electrocardiograms by the identical contour of the complexes which followed the administration of the 2 substances. Of particular in-

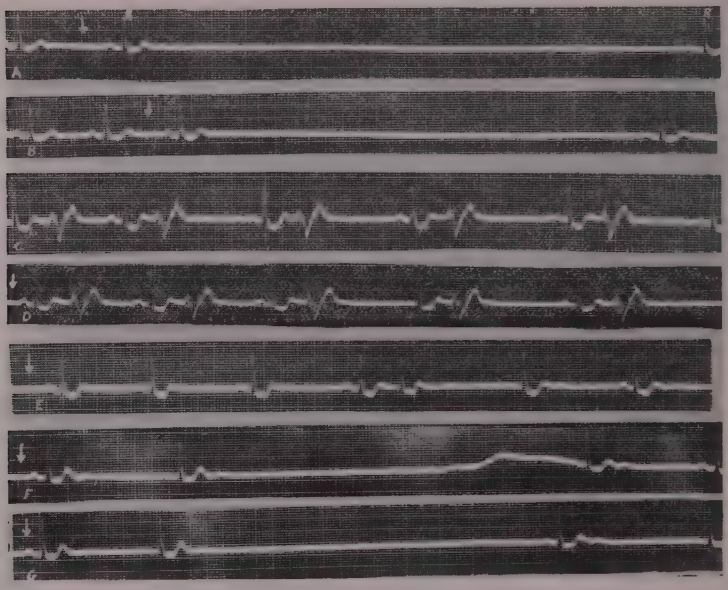

FIG. 2.

(Patient G. S.) Strip A shows cardiac standstill of 7.2 seconds induced by pressure on the right carotid sinus (arrow). Lower strips taken at 15-minute intervals following the oral administration of 60 mg. of parahydroxyphenylisopropylamine hydrobromide. Strips C and D show the standstill prevented by the development of beats of auricular and ventricular origin. In strip E the beats are all supraventricular in origin.

terest was the fact that no symptoms referable to stimulation of the central nervous system were noted. Symptoms of nervousness, tremor, apprehension and sweating were looked for but were not observed in any instance. A moderately severe headache occurred in one case apparently due to the rise in blood pressure.

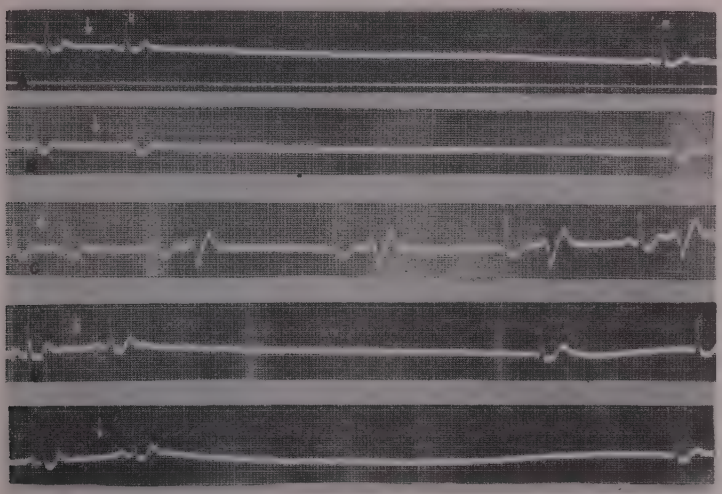

FIG. 3.

(Patient G. S.) Strip A shows a cardiac standstill of 7 seconds induced by pressure on the right carotid sinus (arrow). Strip B taken 30 minutes after the oral administration of 100 mg. of ephedrine sulphate. The cardiac standstill can still be induced. Strip C taken 45 minutes after the drug shows the standstill prevented by the development of beats of auricular origin alternating with beats of ventricular origin. D and E taken at 60 and 75 minutes after the drug show the disappearance of the effect. Compare with Fig. 2 and note that the response to the ephedrine is qualitatively similar but distinctly less intense.

Conclusions. 1. Parahydroxyphenylisopropylamine, when administered by mouth, is effective in the prevention of cardiac arrest. 2. This substance is more active than ephedrine on induced cardiac arrest. 3. When administered in a dosage effective in preventing cardiac standstill, parahydroxyphenylisopropylamine does not produce a central nervous stimulation with resultant unpleasant side effects.

9081 P

Action of Drugs on Electrical Responses of Slowly Conducting Nerve Fibers.

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It has been shown[1] that faradic stimulation of the attached mesenteric nerves causes inhibition of the movements of excised segments of intestine, and that this effect of electrical stimulation can be prevented by cocaine HCl, 1:50,000, atropine sulfate, 1:1000, nicotine, 1:1000, or ephedrine HCl, 1:10,000. In an effort to explain these actions of the drugs named, we observed their effects on action potentials of splanchnic and vagus nerves of rabbits and the sciatic nerves of frogs. Mammalian nerves were immersed in oxygenated modified Locke's solution at 34°C., frog nerve in oxygenated Ringer solution at room temperature. Measured amounts of stock solutions of the drugs were added to the bath. Action potentials of the nerves were recorded by means of a cathode ray oscillograph and appropriate vacuum tube amplifier. Stimulation was by a single dry cell and Harvard inductorium. Electrodes were of platinum. To obtain a record, a nerve was removed from the bath and laid across the electrodes. Records were made before the nerve was drugged, at intervals after adding the drug, and again after allowing recovery of the nerve in fresh Locke's solution. Attention was paid particularly to the "C" wave, since this was thought to represent that type of fibres responsible for intestinal inhibition. The wave was recognized by stimulation required, by amplitude of the wave and by its rate of conduction along the nerve.

Thirty to 45 minutes treatment with cocaine HCl 1:40,000 to 1:20,000 or by atropine sulfate 1:1000 or nicotine 1:1000 resulted in disappearance of the "C" wave (31 tests). This was interpreted to mean that non-medullated and finely medullated nerve fibres were paralyzed by such treatment. Return to fresh bath permitted recovery. Nicotine 1:5000 failed to change the action potential, but atropine sulfate in similar concentration caused disappearance of the C wave in one of 3 nerves tested. Ephedrine HCl 10^{-4} abolished the C wave of 2 nerves, decreased the rate of conduction and amplitude of the wave of 4 nerves and failed to alter one nerve. In higher concen-

[1] Hendricks and Thienes, Proc. Soc. Exp. Biol. and Med., 1931, **28**, 993.

tration (1:1000 to 1:3000) 8 nerves were paralyzed, 9 slightly depressed and 2 unchanged.

Conclusions. Very dilute concentrations of cocaine HCl depress slowly conducting nerve fibres; in higher concentration, a similar depression is produced by ephedrine HCl, atropine sulfate or by nicotine base.

9082 P

Effect of Cortical Extract on Glucose Tolerance of Adrenalectomized and Hypophysectomized Rats.

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In previous work[1] we found that the ability of the hypophysectomized rat to remove excess sugar from the blood stream decreased with time after operation. This gradual decrease in ability to remove excess sugar indicated that the effect must be due to the progressive atrophy or derangement of some other organ following removal of the pituitary body. We gave our first attention to the rôle of the adrenal gland in this effect since Leloir[2] had shown a decrease in the rate of removal of glucose from the blood in adrenalectomized dogs, associated with decreased glycogen formation.

To test this possible connection the following groups of male rats were given 0.125 gm. glucose per 100 gm. body weight by injection of 50% solution into the saphenous vein:

a. Normal control animals.

b. Normal animals injected with 0.2 cc. Wilson's adrenal cortex extract per day.

c. Young adult animals adrenalectomized 6 days previously.

d. Similar adrenalectomized animals injected with 0.2 cc. extract per day for the previous 4 days.

e. Hypophysectomized animals, operated on 17 days previously.

f. Similar hypophysectomized animals injected with 0.2 cc. extract per day for the previous 6 days.

All animals were fasted for 24 hours before receiving the glucose

1 Samuels, L. T., and Ball, H. A., *Endocrin.*, in press.

2 Leloir, L. F., *Suprarrenales y Metabolismo de los Hidratos de Carbono*, Buenos Aires, 1934.

injections. Blood sugar samples were taken from the tail vein, the first few drops being discarded. Samples were taken fasting and at 10, 20, 30, 60, and 120 minutes after injection. Table I gives the average values for the various groups.

TABLE I.

Group	No. Animals	Blood Sugar in mg. per 100 cc. after injection of 125 mg. glucose/100 gm. wt. Fasting	10 min.	20 min.	30 min.	60 min.	120 min.
a. Normals	23	81±1.7	272±4.7	208±2.7	161±2.7	119±1.8	97±1.4
b. Normals, injected	6	88±2.9	269±2.2	206±4.3	157±4.8	120±3.5	97±2.1
c. Adrenalectomized	10	75±3.3	252±9.1	237±4.1	212±4.1	157±2.9	98±2.1
d. Adrenalec., injected	9	78±2.7	268±9.8	213±7.8	157±5.7	110±3.3	94±2.9
e. Hypophysectomized	18	68±2.7	301±8.6	271±6.5	242±9.9	173±4.3	105±2.9
f. Hypoph., injected	6	58±3.0	307±9.5	258±6.8	230±12.7	205±7.0	91±5.3

The defect in ability to remove excess sugar from the blood in the hypophysectomized male rat is not relieved by cortical extract, while that observed in the adrenalectomized animal disappears. It seems then that the deficiency brought on by removal of the pituitary gland is not solely, if at all, a result of atrophy of the adrenal cortex. Since the normal rats injected with cortical extract showed no greater rate of glucose removal than the uninjected controls, the action of the cortical extract in adrenalectomized rats on the tolerance must be due to its specific action in relieving cortical insufficiency.

9083

Inability of Sheep to Develop Antihormone to the Gonadotropic Hormone from Sheep-Pituitary Glands.*

K. W. THOMPSON. (Introduced by Harvey Cushing.)

From the Laboratories of Physiology and Surgery, Yale School of Medicine.

Information concerning antihormones does not as yet indicate whether these substances are immune bodies or chalones (hormone antagonists, Sharpey-Schafer). Most observers are agreed that antihormones may be secured in serum after suitable injections of pituitary extracts into animals, as was first described by Collip and his co-workers.[1] Meanwhile, attention has been called to the pres-

* The experiments were aided by a grant from the Josiah Macy, Jr. Foundation.

[1] Anderson, E. M., and Collip, J. B., *Lancet*, 1934, **1**, 76.

ence in the injected extracts of proteins which may be antigenic in the recipient.[2, 3] That certain active protein principles, such as the enzymes, pepsin, and steapsin, are antigenic in heterologous species is well known, and it is conceivable that hormones, which are substances closely related to enzymes, may also be antigenic under certain circumstances.

The purpose of the present communication is to record observations which appear to support the concept that antihormones are the product of the reaction of an animal to an antigen. In these experiments an extract of sheep-pituitary glands, which had produced gonadotropic antihormone in several species of animals, did not produce this antihormone in sheep.

It will be recalled that Twombly,[3] working along somewhat similar lines, found that prolan injected into rabbits produced an antihormone, but he found no evidence that it was present in the sera of patients, even after prolonged injections. Experiments with parabiosis in rats[4, 5] and with implants of rat pituitaries into rats have also failed to show evidence of formation of antihormones in these animals by these methods. The first report of failure to produce antihormones by injections of a thyrotropic extract into a foreign species was made by Werner,[6] who found that injections of a bovine thyrotropic flavianate did not produce antihormones in guinea pigs. With these exceptions the antihormone studies so far reported have concerned injections of the commonly available bovine and sheep-pituitary products, pregnant mare serum, or human pregnancy-urine preparations into the usual laboratory animals, in which heterologous pituitary proteins must be considered.

The present experiments were devised to determine whether antihormones could be produced in the sera of sheep by injections of highly active sheep-pituitary extracts, which were known to produce antihormones readily in other species of animals. Brief descriptions of the extracts employed follow.

Extract No. 1 was prepared by alkaline extraction of fresh sheep-pituitary glands with subsequent alcohol precipitation at a pH of 5.6, using a method described by VanDyke and Wallen-Lawrence.[7]

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2 Bachman, C., PROC. SOC. EXP. BIOL. AND MED., 1935, **32**, 851.

3 Twombly, G. H., *Endocrinology*, 1936, **20**, 311.

4 DuShane, G. P., Levine, W. T., Pfeiffer, C. A., and Witschi, E., PROC. SOC. EXP. BIOL. AND MED., 1935, **33**, 339.

5 McCahey, J. F., Solway, D., and Hansen, L. P., *Pennsylvania M. J.*, 1936, **39**, 223.

6 Werner, S. C., PROC. SOC. EXP. BIOL. AND MED., 1936, **34**, 390.

7 VanDyke, H. B., and Wallen-Lawrence, Z., *J. Pharm. and Exp. Therap.*, 1933, **47**, 163.

For the purpose of this communication it is sufficient to state that the extract was rich in gonadotropic and thyrotropic hormones. The protein of the extract may well have been partially or completely denatured by the exposure to alcohol. This extract has aroused gonadotropic antihormone in rats, guinea pigs, rabbits, dogs, monkeys, and a horse.

Extract No. 2 was prepared by alkaline extraction from an acetone-dried powder of whole sheep-pituitary glands. This extract also was rich in thyrotropic and gonadotropic hormones. In this instance, although acetone is said to be less likely to denature proteins than alcohol, some denatured protein may have been present. Injections of this extract have been followed by the formation of antihormones both in rats and dogs.

Two sister ewes 4 months old were used for the experiment, and injections were started after samples of their sera had been taken for control tests. Ewe No. 1 was injected subcutaneously each day with 25 cc. of extract No. 1, and ewe No. 2 was injected with the same amount of extract No. 2. The animals were bled at intervals, and tests of each specimen of serum were made upon immature rats. After 6 months of injections, when it would appear from previous experience that antihormones had been given adequate time to develop, the animals were sacrificed. Hypertrophy of the external genitalia and signs of sexual stimulation, which appeared shortly after the injections were started, remained during the period of the injections. Within the period of treatment an area of skin on each ewe's back was shaved, and the fact was noted that the wool grew normally. Neither ewe developed hypercholesterolemia.

The power of the sera to modify the gonadotropic effects of the 2 pituitary extracts was tested in 25-day-old immature rats. The standard activity of each extract was determined by a hundred-hour test in which the rats of each group were given 3 subcutaneous injections each day for 2 days, and the animals were sacrificed one hundred hours after the first injection of the extract.

Another group of rats was injected with serum twice daily beginning 2 days before the hundred-hour period and continuing through the hundred-hour period. The object was to have the serum in the process of absorption during the action of the injected gonadotropic hormone. The average weight of the ovaries of each group of rats was used as a measure of the gonadotropic effect.

Study of the results revealed no evidence to indicate that gonadotropic antihormones were produced in either of the sheep by the injections. Two of the specimens of serum were tested in July and

August during days of extreme heat, and these tests (starred in the table) were unsatisfactory because the rats grew poorly and the ovaries of the controls did not respond adequately to the stimulation of the gonadotropic hormone. The other tests were entirely satisfactory. The results are shown in Table I.

TABLE I.
The Average Weights of Ovaries of Rats Tested with Sheep-Pituitary Extracts and with the Sera of 2 Sheep which Received Prolonged Injections of Sheep-Pituitary Extracts.

	The day serum was taken				
	0 day	85th day*	115th day*	145th day	180th day
Controls; no serum; Extract No. 1, 0.5 cc.	59	32	24	54	50
Serum, Ewe No. 1, 0.5 cc. plus Extract No. 1, 0.5 cc.	61	16	23	38	50
Serum, Ewe No. 2, 0.5 cc. plus Extract No. 1, 0.5 cc.	63	16	33	44	38

Summary. Sheep-pituitary extracts, which had produced gonadotropic antihormones in several species of animals, were injected into 2 sheep for 6 months, during which time no gonadotropic antihormone was found in the sheep-sera.

9084

Non-Specificity of Thyrotropic Antihormone.*

K. W. THOMPSON. (Introduced by Harvey Cushing.)

From the Laboratories of Surgery and Physiology, Yale School of Medicine.

The preparation of an antiserum which inactivated pituitary gonadotropic hormone of several species of animals, including man, has been reported.[1] The present study of the same serum was made in order to extend the observations to include its effect upon pituitary thyrotropic hormone from several species of animals. Along somewhat similar lines, Gregerson, Clark and Kurzrok[2] injected prolan into rabbits and prepared an antiserum which inhibited the gonadotropic activity of an extract of bovine pituitary glands. Prior

* The experiments were aided by a grant from the Josiah Macy, Jr. Foundation.

1 Thompson, K. W., and Cushing, H., *Proc. Roy. Soc., London*, s.B., in press.

2 Gregerson, H. J., Clark, A. R., and Kurzrok, R., PROC. SOC. EXP. BIOL. AND MED., 1936, **35**, 193.

to the appearance of these reports, the information available indicated that an antihormone preparation inactivates only the extract that arouses the antagonistic response (Bachman, Collip and Selye).[3]

Briefly, the tests were devised to determine whether the standard effects of injections of the several pituitary thyrotropic hormone preparations into test animals could be modified by injections of the antiserum.

The thyrotropic extracts used in the tests were the following: (1) a sheep-pituitary extract prepared by a method described by VanDyke and Wallen-Lawrence[4]; (2) an alkaline extract of an acetone-dried powder of whole sheep-pituitaries; (3) a bovine thyrotropic extract kindly contributed by Dr. Arnold Loeser; (4) a thyrotropic extract also of bovine pituitaries supplied by Parke, Davis and Company; (5) an extract of the combined urine samples of 3 men that had complete myxoedema following total thyroidectomy.†

The serum was obtained from a mature, female collie dog, which had been injected daily for 16 months with extract No. 1. She showed marked signs of hypothyroidism, namely, obesity, sluggishness, coarse fur, and hypercholesterolemia. Her thyroid gland, as disclosed at biopsy, was activated shortly after injections were started, and it subsequently became completely inactivated. Signs of sexual stimulation, which also appeared shortly after injections were started, rapidly disappeared and did not reappear.

The standard effects of each thyrotropic extract were determined in young male guinea pigs which were subcutaneously injected with the extract twice daily for 3 days and were autopsied on the fourth day. For comparison with these animals, other groups of guinea pigs were given the same tests of the thyrotropic extracts together with injections of the antiserum, which were given twice daily for 2 days before and during the administration of the thyrotropic hormone. Control experiments were made with serum alone. The thyroid glands of the animals were weighed, fixed in Zenker's fluid, and sections were stained with hematoxylin and eosin. The

[3] Bachman, C., Collip, J. B., and Selye, H., PROC. SOC. EXP. BIOL. AND MED., 1934, **32**, 544.

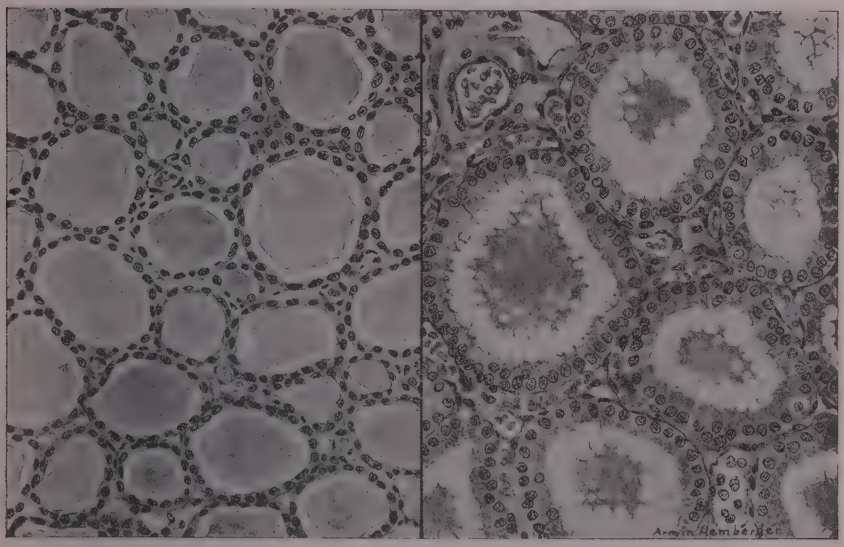
[4] VanDyke, H. B., and Wallen-Lawrence, Z., *J. Pharm. and Exp. Therap.*, 1933, **47**, 163.

† The operations for total thyroidectomy were performed by Dr. Elliott C. Cutler of the Peter Bent Brigham Hospital. Not one of these patients had taken thyroid in any form within a year prior to the time the urine was tested, and each had the classical signs and symptoms of myxoedema. The extracts were made by precipitation of the urine with 80% acetone.

histological signs which were used in comparison to the control findings for estimation of the thyrotropic effects were diminution in the amount of stainable colloid, elevation and proliferation of the epithelium of the acini, and increased vascularity.

Special precautions were taken to have the guinea pigs' cage at a constant temperature of 29°C. Two weeks before testing, the animals were placed in this cage where they remained until autopsied. Such regulation of temperature has been reported by Englemann[5] and by Schoeller[6] to be an aid in securing uniformity of results. The further precaution was taken to inject the serum into one side of the guinea pigs and the extract into the other side, in order to prevent mixture of the materials at the site of injection.

Study of the average weights of the thyroid glands and the histological preparations revealed that injections of the dog's serum into guinea pigs produced an effect resembling hypophysectomy, and substantial doses of each of the thyrotropic preparations were inactivated in these test animals by the antiserum. The results are illustrated in Fig. 1 and are listed in Table I.



A B

FIG. 1.

A. Thyroid gland of guinea pig treated with serum and Extract No. 3.
B. Thyroid gland of guinea pig treated with Extract No. 3 alone.

[5] Engelmann, B., *Arbeitsphysiol.*, 1930, **2**, 387.

[6] Schoedel, W., *Arch. f. exp. Path. u. Pharmakol.*, 1933, **173**, 314.

TABLE I.

Inactivation of Thyrotropic Hormone from Different Species of Animals Produced in Guinea Pigs by the Serum of a Dog which Had Been Injected for Sixteen Months with a Sheep-Pituitary Extract (No. 1).

Source of Hormone	Thyroid glands of guinea pigs: Hormone alone Wt.	Activity	Thyroid glands of guinea pigs: Hormone plus Serum Wt.	Activity
Extract No. 1 (Sheep), 1.0 cc.	56	Activated 2 plus	24	Inactivated
Extract No. 2 (Sheep), 0.5 cc.	81	" 3 "	25	"
Extract No. 3 (Beef), 0.5 cc. (Loeser)	75	" 3 "	17	"
Extract No. 4 (Beef), 1.0 cc. (P. D. & Co.)	96	" 4 "	47	Partially inactivated
Extract No. 4 (Beef), 0.25 cc. (P. D. & Co.)	63	" 3 "	29	Inactivated
Extract No. 5 (Man), 3.0 cc.	25	" 1 "	25	"

Summary. Daily injections of sheep-pituitary extract into a dog produced an antihormone which inactivated in guinea pigs substantial test doses of thyrotropic hormone from sheep and bovine pituitary glands and from human urine of myxoedema. Injections of the serum alone into guinea pigs produced upon their thyroids an effect which resembled hypophysectomy.

9085

The Augmentary Factor in Animal Sera After Injections of Pituitary Extract.*

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From the Laboratories of Surgery and Physiological Chemistry, Yale School of Medicine.

There has recently appeared a most timely report of the preparation of a pituitary gonadotropic antagonist by Evans, Korpi, Pencharz, and Simpson.[1] According to these workers this pituitary fraction, representing what Sharpey-Schafer would have called a chalone, is probably present in considerable amounts in most crude pituitary extracts, and accounts for the strange lack of activity shown by certain gonadotropic extracts from which greater potency would have been expected. Such an extract has been the subject of inves-

* The experiments were aided by a grant from the Josiah Macy, Jr. Foundation.

[1] Evans, H. M., Korpi, K., Pencharz, R. I., and Simpson, M. E., *Univ. of Calif. Pub. in Anat.*, 1936, **1**, 237.

tigation in this laboratory in experiments where the development of gonadotropic antihormones in animal sera was studied during many weeks of its injection.

The purpose of the present communication is to record the facts which lead to the following conclusion, *viz.*, that the results of the experiments are best explained by assuming the formation in the injected animal of an antihormone to the antagonist.

The injected extract† was prepared from sheep-pituitary glands by a method slightly modified from that described by VanDyke and Wallen-Lawrence.[2] The animals injected were 2 large dogs, a male and a female, and a young mare. They received the following daily dosage of the extract: the male dog, 5 cc.; the female dog, 25 cc.; and the mare, 50 cc. The animals were bled before the injections were started, after which time the dogs were bled every week, and the mare approximately every 20 days.

The sera were each tested in 25-day-old rats for ability to modify the gonadotropic effects of the extract, as follows: (1) the standard activity of the gonadotropic extract *alone* was determined by giving to a group of rats 3 subcutaneous injections of the extract on each of 2 successive days, the rats being sacrificed one hundred hours after the first injection. (2) The tests for the combined effect of serum and extract were coincidentally made in other groups of rats which were given serum twice daily beginning 2 days before the injections of extract were begun and continuing through that period. Care was taken to inject the serum into one side of a rat and the extract into the other side, so as to avoid mixing the materials at the site of injections. The average weight of the ovaries of each group of rats was used as a measure of the gonadotropic effect.

The normal sera had very little effect upon the gonadotropic action of the pituitary extract in the rats. After 20-30 days of injections the serum of each of the 3 animals began to reveal an unsuspected ability to augment the gonadotropic action of the extract. The augmenting power of the sera reached a peak after 50-80 days of injections, at which time the gonadotropic action of the extract was enhanced three-fold. This remarkable power of augmenting gonadotropic action was rapidly lost by both the bitch and the mare after

† For several reasons, some of which were discussed in the report of Evans and his co-workers, the suspicion has long been entertained by us that this extract contained an antagonist in addition to a substance which gave rise to antihormones.

2 VanDyke, H. B., and Wallen-Lawrence, Z., *J. Pharmacol. and Exp. Therap.*, 1933, **47**, 163.

70-90 days of injections, at which time moderate doses of either of these 2 sera completely inactivated the extract. The serum of the male dog, which received the smallest dose of extract, continued to augment the gonadotropic action of the extract after 130 days of injections, when this report was written. The results of the tests are illustrated in Fig. 1. The augmenting serum transformed the rather weak gonadotropic extract into an exceedingly potent one, whose effects on the ovary resembled those of a highly active preparation of the serum of pregnant mares.

The nature of the augmenting action of the sera. This question was immediately raised, and the possible causes of the phenomenon,

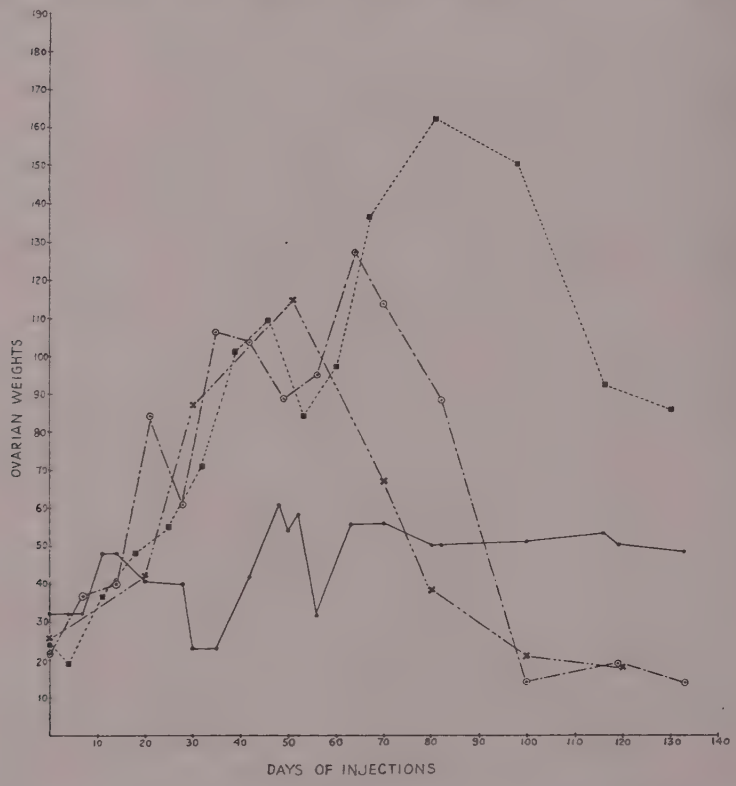


FIG. 1.

The development in the sera of the factor which augmented in test animals the action of pituitary gonadotropic extract. The average weight of the ovaries of rats tested with serum plus extract, and with extract alone, are plotted in relation to the day when serum was taken for each of the combined tests. The dotted lines represent the effect of the sera upon the potency of the extract. The unbroken line represents the potency of the extract alone at the time of each test.

so far as could be seen, were tentatively listed as follows: (1) the presence of F.S.H. in the sera; or (2) a delayed absorption of the gonadotropic hormone with a more gradual action possibly produced by the serum, permitting augmentation in the manner of the tannate or protamines of insulin which supposedly delay absorption and thereby spread the action of the hormone over a greater period of time; or (3) the presence in the sera of an antihormone to the antagonist which, by holding in abeyance both the antagonist in the tested rat and in the injected pituitary extract, gave complete freedom of action to all the gonadotropic hormone.

Further experiments designed to shed light on the subject were made as follows: First, the sera were tested alone as for F.S.H., in hypophysectomized immature rats. These animals were hypophysectomized at 25 days of age, and 5 days later the tests were begun. In no instance were there signs of stimulation of the ovaries; in fact, the tested hypophysectomized rat ovaries tended to be slightly smaller and less active in appearance than those of the normal uninjected controls.

Second, the same tests of the sera alone as for F.S.H. were repeated in normal immature rats. When read at 72 hours, an effect similar to that of F.S.H. was noted; in each animal so tested definite follicles developed in the ovaries, which were nearly twice normal size.

The failure of the augmenting principle contained in the sera to stimulate the ovaries of hypophysectomized immature rats served to differentiate this principle from F.S.H.

In normal immature rats the injected serum apparently released F.S.H. present in the pituitary glands of these animals, since the serum in itself having no pituitary-like action produced an effect identical with that of F.S.H.

What indicated that a delay of absorption of the gonadotropic extract was not responsible for the enhancing effect was the fact that with the technique of the tests remaining the same, the sera produced an increasing augmentation from week to week, and ultimately caused inhibition of the extract rather than augmentation.

An antihormone to the antagonist cannot be excluded at the present time as the cause of the augmentation of the gonadotropic extract by the sera. The following facts appear to support this assumption: (1) the extract injected has readily produced antihormone to the gonadotropic and thyrotropic hormones in several species of animals; (2) the augmenting factor was found only in the pseudo-globulin fraction of the sera, the same fraction that

contains the immune bodies‡ (precipitation between 33% and 50% saturation with ammonium sulfate); (3) the factor in the sera which inactivated gonadotropic hormone and prevented further augmentation, is usually described as an antihormone, and was also found only in the pseudo-globulin fraction; and (4) the sera, which did not act upon the ovaries of the hypophysectomized rats, apparently released follicle-stimulating hormone already present in the animals, so that the normal rat ovaries were stimulated. The same effect would have been produced if the antagonist of Evans and co-workers had been inactivated in these animals.

Whether this supposed antihormone inactivated antagonist of both the rat and the extract injected into the rat, or only that of the rat itself, remains to be investigated. The rat ovary, released from antagonist, and stimulated by F.S.H. from its own pituitary, undoubtedly reacts as if augmented after injections of gonadotropic extracts that are rich in the luteinizing factor and poor in F.S.H. This sort of augmentation by the interaction of these gonadotropic fractions is now well known, and probably was a factor in the present experiments. It would seem, however, that the greater effect of removal of antagonist in the injected extract may also have been effected by the sera, since the augmentation was so marked. It will be recalled that Evans and his co-workers stated that their pituitary extracts were enhanced in activity three-fold by the removal of the antagonist. The sera of our studies at their greatest potency produced the same degree of augmentation.

Summary. Injections of a gonadotropic extract of sheep-pituitary glands into a horse and 2 dogs induced the formation in their sera of a factor which augmented three-fold the gonadotropic activity of the extract when tested in immature rats. The augmenting factor had no gonadotropic action in hypophysectomized immature rats, but produced in immature normal rats an effect resembling F.S.H. or synergist. The augmenting factor, possibly an antihormone to the pituitary gonadotropic antagonist, was present only in the pseudo-globulin fraction of the sera, which fraction also carries the immune bodies and other antihormones.

We are indebted to Parke, Davis and Co., through the courtesy of Mr. G. T. Clark, for the treatment of the mare and the preparation of its serum.

‡ From unpublished experiments by Thompson and White.

9086

Preparation and Assay of Mammotropic Hormone.

WILLIAM R. LYONS.*

From the Division of Anatomy, University of California.

It has been possible to prepare mammotropic hormone with an average minimal effective dose of 0.1 mg. when tested in squabs by means of the 4-day (4 injections) "systemic" test introduced by Riddle, *et al.*,[1] and 0.1γ when tested by means of the 2-day (1 injection) "local" test.[2] This hormone (unheated) does not stimulate the thyroids or gonads of immature squabs, rats or guinea pigs in doses of 100 mg. (4 daily doses of 25 mg.) whereas purified thyrotropin and gonadotropin are effective in doses of less than one mg. Furthermore, hypophysectomized rats show no increase in body weight, nor repair of adrenals, thyroid, gonads, when injected with purified, unheated mammotropin (10) mg. per day for 20 days.

One method of preparing mammotropin is presented here because it yields equivalent amounts of mammotropic and adrenocorticotropic hormones, sufficiently separated to prove them distinct substances.

Grind thoroughly 1 kg. undissected sheep pituitaries (equivalent to approximately 500 gm. anterior lobe). Add 4 litres acetone (to which has been added 100 cc. 35% HCl) one litre at a time, mixing thoroughly by putting the mash through the grinder several times after each addition.

Centrifuge,† and to the supernatant add 5 litres acetone. Agitate forcibly and if possible allow to settle overnight at a low temperature. Dissolve the gummy precipitate in a mixture of 100 cc. H_2O and 100 cc. acetone, and reprecipitate by adding 800 cc. acetone. Dissolve precipitate in 200 cc. H_2O, add 100 cc. NH_4OH (28% NH_3) and allow to stand at room temperature for about 3 hours. Then add 2 volumes of acetone, centrifuge and discard precipitate (or reëxtract it by dissolving in 100 cc. H_2O and adding 50 cc. NH_4OH and 2 volumes of acetone as before). To supernatant(s) add 1 volume acetone and 10 cc. 35% HCl. Agitate forcibly to precipitate.

* Aided by a grant from the Board of Research, University of California.

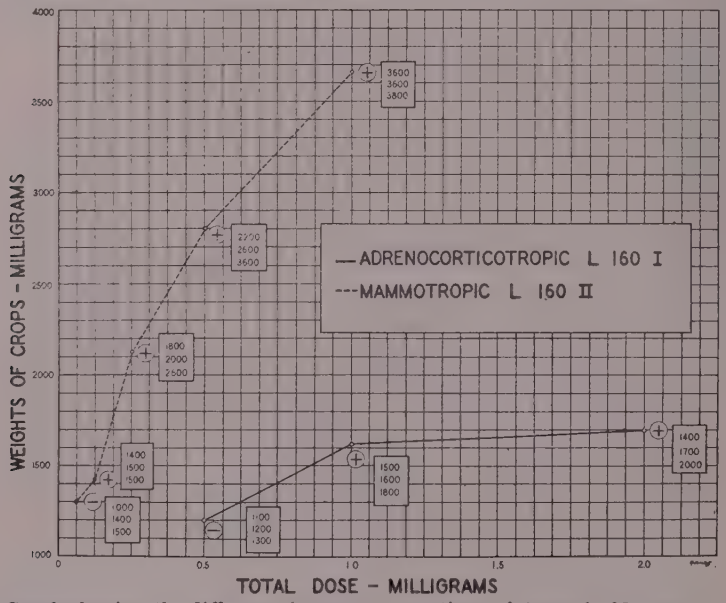
1 Riddle, O., Bates, R. W., Dykshorn, S. W., *Am. J. Physiol.*, 1933, **105**, 191.

2 Lyons, W. R., and Page, E., PROC. SOC. EXP. BIOL. AND MED., 1935, **32**, 1049.

† To increase yield, the mash may be re-extracted with 2 litres 85% acetone plus HCl to pH 1.5 and the supernatants pooled. In the first extraction with absolute acetone, consideration is given to the H_2O in the tissue.

Discard supernatant and evaporate acetone-NH_3 from precipitate. Dissolve in 200 cc. H_2O with sufficient N/1 NaOH to obtain a clear solution. Adjust to pH 6.5 with N/1 HCl and centrifuge out precipitate.‡ Adjust to pH 5.5 and freeze out precipitate. Several adjustments of the supernatant should be made with HCl and NaOH and freezing in order to increase the yield of isoelectric precipitate, which usually approximates 1.0 gm. Dissolve precipitate in as little H_2O and N/1 NaOH as is necessary to give a clear solution at pH 8.0. After ascertaining per cent of organic matter, adjust solution to 1% at pH 7.6, add Hexylresorcinol to 1:15,000 or Merthiolate 1:5000, and sterilize 20 minutes at 100°C. on 2 successive days.

The accompanying graph shows the average weights of the dissected, reactive areas of both crop sacs after injections of the mammotropic (pH 5.5 iso-electric) and adrenocorticotropic (pH 6.5 iso-electric) fractions respectively. Three birds were used at each level, and were sacrificed 24 hours after the fourth daily injec-



Graph showing the difference in crop sac reactions of 1-month-old squabs to 4 subcutaneous, daily injections of different amounts of incompletely separated fractions of mammotropin and adrenocorticotropin.

A + accompanies such positive reactions as fall within the weight range of normal crop sacs (1000-1500 mg.).

‡ This fraction has been investigated by Mr. Henry D. Moon and is the subject of an accompanying paper.

tion. It would appear that the pH 5.5 iso-electric contains about 10 times as much mammotropin as the pH 6.5 iso-electric or adrenocorticotropic fraction. This represents the typical finding in about 50 batches prepared in the above manner.

The "local" intradermal method of testing mammotropin (Fig. 1) while as yet lacking a gravimetric basis, requires less hormone and time than the "systemic" test, and is indispensable in studies of urinary mammotropin (especially that of normal males). The histology of the local reaction is the same as seen in the "systemic" test, but the maximal reactions (Fig. 5) show less growth because of the shorter period (48 hours). The minimal effects are usually limited to circumscribed areas (3-4 cm. in diameter) better seen after tearing the muscle layer off the stretched crop (the fingernail does this nicely). Histologically these areas may show as much

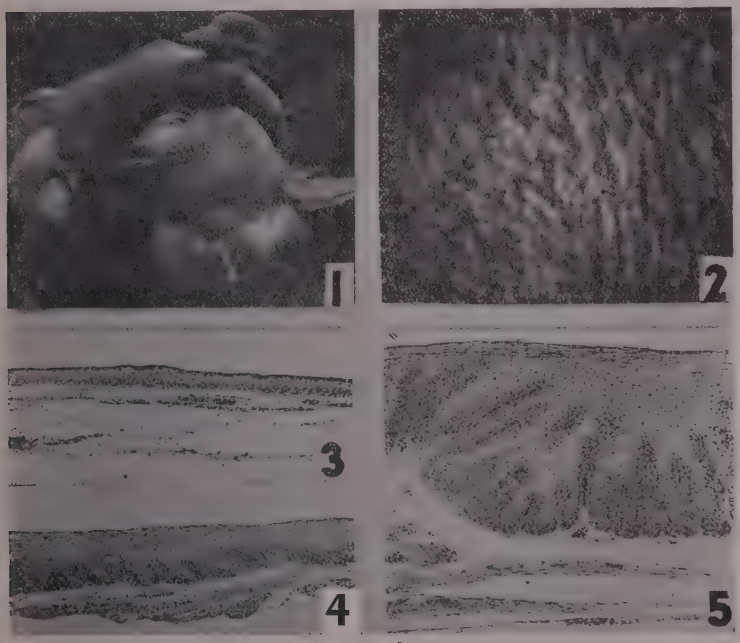


FIG. 1. The plucked crop sac area of a 1-month-old Silver King squab. 0.1 cc. of solution is injected into a feather ridge at the point indicated. A different dilution is tested on the opposite side.

FIG. 2. The appearance of a positive "local" reaction to mammotropin when the stretched crop sac is held to the light. ×2.

FIG. 3. Section of normal crop sac membrane of a 1-month-old squab. × 40.

FIG. 4. Section showing a low grade reaction obtained 48 hours after 1 "local" intradermal injection of 0.1 microgram of mammotropin. × 40.

FIG. 5. High grade "local" reaction to 6.4 micrograms of same. × 40.

growth as seen in Fig. 4 while the surrounding membrane is similar to the crop of control squabs (Fig. 3). The gross appearance of the "local" reaction when the crop sac is stretched in front of a light is shown in Fig. 2. It has been possible to show that round discs 3 cm. in diameter cut with a cork-borer from the reactive area of crop sacs stretched tightly over a cork and washed with water, when desiccated in the 110°C. oven overnight show weights proportional to the amount of hormone injected in the dose range from 0.1 to 10.0 micrograms. For those who prefer a gravimetric unit, this observation may have something to offer, but the qualitative minimal effective dose as is used in tests for the female sex hormone[3] seems sufficient at present. Larger numbers of squabs for each level would have given our results greater statistical significance, but we have not found it economically practical to use more than 5, and we customarily use 3. Our squabs coming from 2-egg nests are chosen one month from hatching for their uniformity, from a large local loft; they are all Silver Kings and may occasionally vary 100% in reactivity to the hormone. Of 5 birds tested on the right side with 0.05γ of L 160 II only one reacted, while 4 reacted to 0.1γ injected on the left side. Only in large doses is a local injection on one side carried over (probably systemically) to affect the opposite sac.

Conclusion. A method has been described for extracting mammotropic hormone from sheep pituitaries involving its solubility in HCl-acetone and NH_3-acetone, and its precipitation with isoelectric protein at pH 5.5. A fraction made by the same procedure but precipitated at pH 6.5 has been shown to contain relatively little mammotropin, and is identified in the following paper as the adrenocorticotropic hormone. The "local" intradermal squab test has several advantages over the "systemic" tests of Riddle, *et al.*, when only mammotropic hormone is to be tested for, but the latter test has the very great advantage of allowing one to assay for the gonadotropic, thyrotropic, adrenotropic and mammotropic hormones on the same bird.

[3] McShan, W. H., and Turner, C. W., PROC. SOC. EXP. BIOL. AND MED., 1936, **34**, 50.

9087

Preparation and Biological Assay of Adrenocorticotropic Hormone.

HENRY D. MOON.* (Introduced by H. M. Evans.)

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Adrenocorticotropic extracts† were tested in normal 21-day-old male rats and in hypophysectomized rats. The amount of adrenocorticotropic hormone necessary to cause an increase of 50% over the adrenal weight of controls when injected into 21-day-old male rats in 3 doses over a period of 3 days was defined as one normal rat unit. A total dose of 20 mg. was usually found to be equal to one unit.

The injections of large amounts of beef brain (1000 mg.) produced no significant change in adrenal weights of normal 21-day-old male rats. Adrenocorticotropic preparations from beef anterior pituitaries showed the same activity as adrenocorticotropic preparations from whole sheep pituitaries, indicating that posterior lobe substances were not causing the hypertrophy of the adrenal cortex in the normal male rats.

Male rats between 40 and 50 days old were hypophysectomized,‡ and at least 30 days were allowed for atrophy of the adrenals before injections were begun. The completeness of the operation was checked by examination of the sella turcica under dissecting binocu-

TABLE I.

The Effect of Adrenocorticotropic Extracts in Hypophysectomized Rats.

Extract	Hypophysectomized Rat	Total Dose of Adrenocorticotropic Hormone, units	Length of Treatment, days	Wt. of 2 Adrenals, mg.	Increase Over Controls mg.	Increase Over Controls %	Wt. of Thyroids, mg.	Body Growth, gm.
M 126	W 63	5.1	10	19.9	9.4	89.5	—	2
	W 44	5.1	10	23.8	12.3	117.1	—	2
	GH 37	5.1	10	21.0	10.5	100.0	—	—8
M 133	BH 8421	5.3	10	22.0	11.5	109.0	9.5	0
	BH 8406	5.8	10	22.8	12.3	117.1	12.5	2
	W 8383	5.8	10	24.5	14.0	133.2	9.0	—3
Controls (7 rats)		0.0	0	10.5 (6.0-13.5)	—	—	13.1 (8.0-17.2)	—

* Aided by grants from the Board of Research, University of California, and the Rockefeller Foundation, New York City.

† Adrenocorticotropic extracts were prepared by the method described by Dr. William R. Lyons in the accompanying paper. The precipitate obtained at pH 6.5 was the corticotropic fraction.

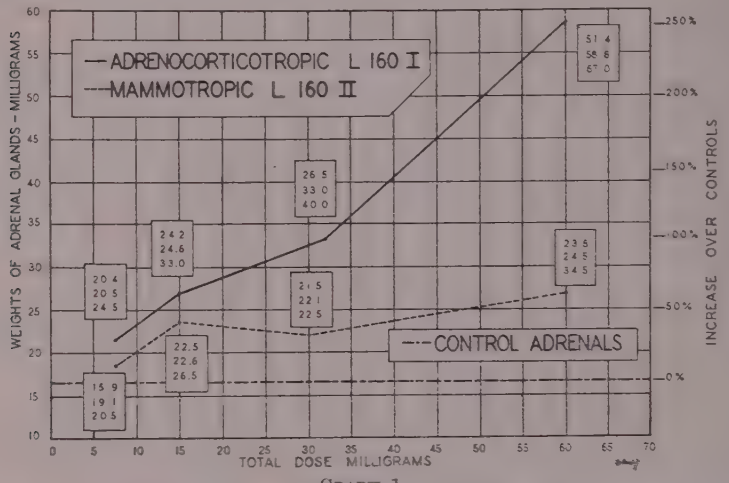
‡ The hypophysectomies were performed by Mr. L. L. Bennett.

lars. Table I shows the effect of adrenocorticotropic extracts in hypophysectomized rats.

From the results obtained in hypophysectomized rats, it can be seen that the adrenocorticotropic preparations contained no growth hormone at the level tested. The thyroids showed no histological evidence of stimulation.

Adrenocorticotropic extracts when injected subcutaneously into squabs for 4 days showed no stimulation of the thyroids or of the testes. The crop glands were stimulated.

Adrenocorticotropic and mammotropic fractions from the same extract were compared at various levels for their adrenocorticotropic potencies in normal 21-day-old male rats. The results are given in Graph 1.



GRAPH 1.
Effect of Adrenocorticotropic and Mammotropic Fractions, Normal 21-Day-Old Male Rats.

The adrenal cortices of normal immature rats injected with adrenocorticotropic hormones§ (Fig. 2) differ markedly from the untreated controls (Fig. 1). There is an *increased* fat content throughout the whole adrenal cortex. In untreated controls there is an intermediate zone between the glomerulosa and the fasciculata in which there is very little or no fat. One of the earliest signs of stimulation is the presence of fat in the cells of this zone. In injected rats there is hypertrophy and hyperplasia of the cortical cells. The hyper-

§ Work in progress indicates that the cortical hypertrophy obtained in immature rats with adrenocorticotropic extracts is not affected by the removal of all thyroid tissue visible under dissecting microscopes.

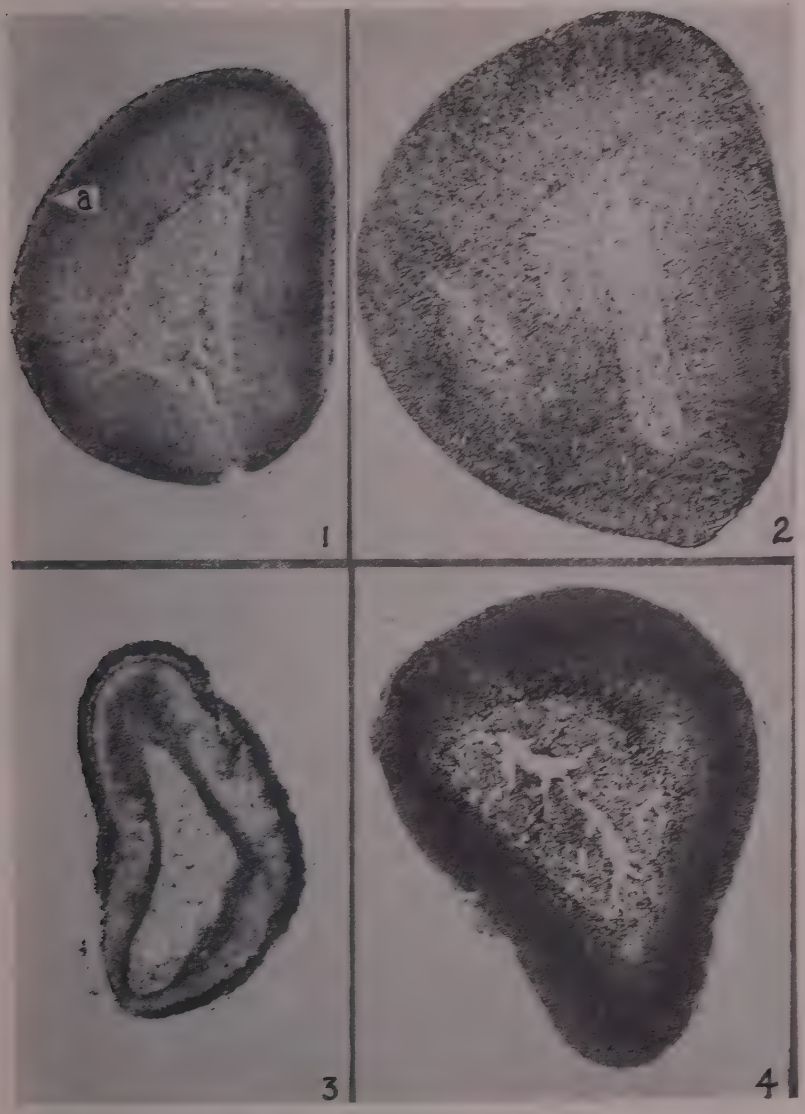


PLATE I.

Adrenals Stained with Sudan Black and Haematoxylin.

FIG. 1. **Untreated normal 24-day-old male rat.** × 20. *a*. **Fat-free zone between the zona glomerulosa and the zona fasciculata.**

FIG. 2. **Normal 24-day-old male rat injected with 2 units of adrenocorticotropic hormone.** × 20.

FIG. 3. **Untreated hypophysectomized rat.** × 20.

FIG. 4. **Hypophysectomized rat injected with 5.8 units of adrenocorticotropic hormone.** × 20.

plasia is limited to the cells of the zona glomerulosa and the outer zona fasciculata. There is a definite hyperemia proportional to the amount of adrenocorticotropic extract injected.

The adrenal cortices of hypophysectomized rats treated with adrenocorticotropic extracts (Fig. 4) show a restoration of the cytoplasm of the cortical cells. The nuclei of the cells are no longer pycnotic. There is a great increase in the fat content of the cortical cells.

9088 P

Studies on the Enumeration of Marine Anaërobic Bacteria.

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Considerable difficulty has been experienced in estimating the abundance of marine anaërobic bacteria although they have been demonstrated[1] in nearly all samples of water or mud examined. Most of the conventional procedures[2] such as the incubation of plates in anaërobic jars have failed to yield reproducible results and moreover, the use of such complicated, space- and time-consuming apparatus is entirely impracticable aboard a rolling boat at sea. The application of oval tubes as described by Anderson[3] for the enumeration of anaërobes has exceeded expectations.

Ordinary round glass tubes with sealed ends as used by Roux[4] and Burri[5] are satisfactory for the cultivation of anaërobes but the curvature of the glass makes it virtually impossible to count the colonies which develop, particularly when it is necessary to use a hand lens. The Kimball Glass Company fabricated special oval tubes for us with flat, parallel sides, thereby obviating this difficulty. The oval tubes are 6x14 mm. in cross-section and 380 mm. long with one end permanently sealed and the other end flared to facilitate the introduction of the medium. The tubes are sterilized in a pipet can. A test-tube containing 10 cc. of nutrient agar recently heated to nearly 100° C. to expel oxygen and cooled to 45° is inoculated

[1] ZoBell, C. E., and Anderson, D. Q., *Am. Assn. Petrol. Geol.*, 1936, **20**, 258.

[2] Hall, I. C., *J. Bact.*, 1929, **17**, 255.

[3] Anderson, D. Q., In press.

[4] Roux, E., *Ann. de l'Inst. Past.*, 1887, **1**, 49.

[5] Burri, R., *Centralbl. f. Bakt.*, Abt. I, Orig., 1902, **8**, 533.

with the proper dilution of the sample to be analyzed for anaërobes. Without undue agitation (shaking has been found to be unnecessary to insure an even distribution of bacteria) the inoculated medium is poured into the oval tube. As soon as the medium has solidified, it is covered with a deep layer of reduced methylene-blue agar. The seal excludes oxygen and indicates the degree of anaërobiosis, Hall[6] having shown that conditions are suitable for the growth of anaërobes in an environment in which methylene blue remains colorless. The inoculated tubes are then incubated at the desired temperature and they may be observed from time to time without interrupting the experiment. The colonies are counted in reflected light against a dark background. The technic is applicable not only to the enumeration of total anaërobes, but by employing appropriate differential media, the abundance of various physiological types can be estimated. The oxidation-reduction potential of any stratum can be measured by the insertion of long slender microelectrodes.

In several series of 50 duplicate determinations in which the oval-tube count was as low as 30 per cc., the standard deviation was only 7%. This is practically the same as the standard deviation obtained in plate-counts on aërobes. The standard deviation of the anaërobic-jar count was 2 or 3 times as great.

Besides being more reproducible than other methods of determining anaërobes, the oval tube count is more representative of the anaërobic population. Tests on picked colonies revealed that all of the bacteria which develop in the oval tubes are either strict or facultative anaërobes, whereas due to incomplete anaërobiosis, many of the colonies which develop in anaërobic jars prove to be either microaërophiles or even strict aërobes.

Oval-tube counts on samples of sea water collected from various depths were between 10 and 20% as high as the total plate-counts (aërobic). A larger percentage of viable bacteria in bottom muds were anaërobes, the proportion of anaërobes to aërobes increasing with core-depth. Many of the bacteria from the mud which developed in oval tubes proved to be strict anaërobes.

[6] Hall, I. C., *J. Bact.*, 1920, **6**, 1.

9089 P

Effect of Diphtheria Toxin Upon Vitamin C in Adrenals of Guinea Pigs.

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Interest in the effect of seasonal variations in the diet of guinea pigs upon their susceptibility to bacterial toxins led to an extensive study[1] of the metabolism of vitamin A in these animals. The results indicated the participation of some other factor or factors in the increased variations in susceptibility which occur during the winter months. Published reports[2, 3] directed our attention to vitamin C.

The guinea pigs used in the experiments were bred by this laboratory and weighed from 230 to 280 gm. They had been fed the routine winter diet: alfalfa hay, oats, barley, commercial rabbit pellets, cabbage, carrots and mangels, and sodium chloride and water *ad libitum*. During May, occasional supplements of grass were fed.

The adrenals of 10 guinea pigs injected early in December with a uniform lethal dose of diphtheria toxin were examined for their vitamin C content by the method of Bessey and King.[4] One of 10 similar animals which had received one-half the dose of toxin, and one uninjected control were destroyed as each in the first group died. The average vitamin C content of the adrenals of the guinea pigs which had died from the effects of diphtheria toxin was reduced to less than 15% of that of the control animals. The change was much less in the adrenals of the 10 animals that received half the lethal dose of toxin. The average amount of vitamin C was 85.5% of that of the normal controls. In fact, the adrenals of the 5 which were destroyed approximately 48 hours after injection showed an increase in the vitamin C content, and it was not until 24 hours later that a diminution became apparent.

Although the slightly higher vitamin C content of the adrenals of the animals in the second group at the end of 48 hours might have been due to errors of random sampling, the possibility that the toxin, when not given in overwhelming doses, stimulated the ad-

[1] Torrance, C. C., *Am. J. Hyg.*, 1936, **23**, 74.

[2] Harde, E., *C. r. Acad., d. sc.*, 1934, **199**, 618.

[3] Jungeblut, C. W., and Zwemer, R. L., PROC. SOC. EXP. BIOL. AND MED., 1935, **32**, 1229.

[4] Bessey, O. A., and King, C. G., *J. Biol. Chem.*, 1933, **103**, 687.

renals to mobilize ascorbic acid seemed worth investigating. Accordingly, the following May, a group of animals were injected with one-third of the lethal dose of toxin. Five additional uninjected guinea pigs of the same weight, sex, and dietary history were used as controls. Four of the test animals were destroyed 24 hours after injection. There were marked subcutaneous edema and congestion at the site of injection but no gross changes in the adrenals. Chemical examination of these organs indicated that the average vitamin C content was 60% greater than that of the controls. A

TABLE I.

Vitamin C Content of Adrenals of Guinea Pigs Which Had Received One-third of a Lethal Dose of Diphtheria Toxin and Were Sacrificed at Various Intervals.

No. of animals	Survival time, hrs.	Vitamin C content of adrenals			Deviation of average from normal %	Gross physical reactions to toxin	
		Max. mg.	Min. mg.	Aver. mg.		Site of injection	Adrenals
5	controls	.070	.012	.032±.006	0		
4	24	.077	.030	.051±.005	+61.2	marked edema	normal
4	48	.057	.047	.052±.002	+63.4	very marked edema	slight congestion
5	58	.069	.028	.052±.005	+63.8	" " "	" "
4	72	.064	.030	.048±.004	+51.2	" " "	congestion
4	144	.150	.081	.117±.009	+269.5	necrosis	normal

comparison of test animals sacrificed 48 and 58 hours after injection indicated a still further, although slight, increase in this factor. At 72 hours, however, the vitamin C had declined but had not reached the level present in the control group. The 4 remaining test animals were killed on the sixth day. There was a reparative reaction around a central area of necrosis at the site of injection, and the adrenals presented a normal appearance. The average vitamin C content of the adrenals was 270% more than that of the control guinea pigs at the beginning of the experiment.

These results were duplicated when guinea pigs injected intradermally with 3 skin-test doses of diphtheria toxin were killed at daily intervals and their adrenals titrated for vitamin C.

9090

Some Effects of Iodine Given to Rabbits After a Period of Cholesterol Feeding.*

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In rabbits with hypercholesterolemia resulting from long-continued cholesterol feeding, the administration of potassium iodide caused a marked rise in the blood cholesterol.[1] A possible explanation for this rise was that the iodide caused a mobilization of cholesterol in the blood with a corresponding decrease of cholesterol in the tissues, roughly comparable to the effect of the parathyroid hormone on calcium.

To test this hypothesis, the liver cholesterol of cholesterol-fed rabbits with and without the administration of potassium iodide was determined.

The liver was selected for analysis because of the following observation: When rabbits are killed immediately after a prolonged period of cholesterol feeding widespread lipoid infiltration is usually evident including atheromatous patches in the aorta and gross changes in the liver, spleen, adrenals and kidneys. If, however, the cholesterol feeding is discontinued for several months before the animals are sacrificed, the aortic lesions persist but changes in the other organs are less frequent and less marked when present. This is particularly noticeable in the liver and suggests that the lesions in that organ, contrary to those in the aorta, are reversible. Furthermore, Meeker, Kesten and Jobling[2] have recently shown that iodine did not decrease the cholesterol content of atheromatous aortas in rabbits.

Dutch belted rabbits approximately 6 months old and averaging 2 kg. in weight were used. They were kept in individual cages indoors and given a diet of oats and fresh vegetables. Cholesterol determinations[3] were made on whole blood weekly.

A total of 72 rabbits was used. The sex ratio was approximately equal. Sixty-two of the animals were fed one gm. of crystalline cholesterol mixed with the grain 3 times a week. Seventeen of these failed to develop a hypercholesterolemia and were discarded.

* Aided by a grant from the Josiah Macy, Jr., Foundation.

1 Turner, K. B., and Bidwell, E. H., *J. Exp. Med.*, 1935, **62**, 721.

2 Meeker, D. R., Kesten, H. D., and Jobling, J. W., *Arch. Path.*, 1935, **20**, 337.

3 Bloor, W. R., Pelkan, K. F., and Allen, D. M., *J. Biol. Chem.*, 1922, **52**, 191.

The rabbits were divided into 4 groups as follows:

Group I. Controls. Killed after 2 months (Table I).

Group II. Cholesterol fed for 2 months and killed (Table II).

Group III. Cholesterol fed for 2 months, kept on a normal diet for a third month and killed (Table III).

Group IV. As in Group III except that during the third month one gm. of potassium iodide was given 3 times a week (Table IV).

At autopsy the aorta was examined for atheromata. The adrenals were removed and weighed at once. The liver was dissected out, cut into small pieces and dried to a constant weight in an oven at 70 to 80°C. A portion of the dried material was pulverized and stored

TABLE I.
Normal Rabbits Used as Controls.

Rabbit No.	Blood Cholesterol mg. per 100 cc. Range	Aver.	Total Liver Cholesterol, mg.	Cholesterol per 100 gm. Dried Liver, gm.	Weight of Adrenals mg.
341	103-126	113	281	1.56	400
342	96-114	104	229	1.19	400
343	135-156	145	260	1.67	380
344	105-129	118	217	1.43	150
345	110-134	125	222	1.41	390
346	109-124	116	226	1.28	350
355	93-106	100	231	1.35	230
358	119-145	132	159	1.40	190
359	96-128	108	205	1.52	240
360	99-124	112	154	1.39	240
	Aver.	117	218	1.42	297

TABLE II.
Rabbits Fed Cholesterol for Two Months.

Rabbit No.	Blood Cholesterol mg. per 100 cc. Range	Aver.	Total Liver Cholesterol, mg.	Cholesterol per 100 gm. Dried Liver, gm.	Weight of Adrenals mg.
264	138-887	527	942	4.81	1420
262	130-725	482	1475	5.57	1420
312	115-403	347	645	6.33	360
287	111-443	303	1182	6.25	470
314	158-305	265	1930	12.37	320
273	132-325	246	445	2.64	420
331	126-333	204	344	2.16	220
261	110-305	203	948	5.61	910
320	125-245	199	1280	5.52	590
278	108-227	183	391	1.96	830
296	113-217	169	446	2.79	380
329	114-206	167	397	2.66	200
266	93-238	158	750	3.79	510
289	107-205	154	728	2.62	320
304	111-198	150	417	2.36	260
	Aver.	250	821	4.50	575

in a desiccator. For analysis, about 2 gm. of the powdered liver were weighed out, placed in a volumetric flask, and the total chol-

TABLE III.

Rabbits Fed Cholesterol for Two Months and Kept on a Normal Diet for a Third Month Before Killing.

	Blood Cholesterol mg. per 100 cc.					
		Aver.				
Rabbit No.	Range	Feeding Period	3rd Month	Total Liver Cholesterol, mg.	Cholesterol per 100 gm. Dried Liver, gm.	Weight of Adrenals, mg.
285	167-834	557	423	772	3.86	1030
279	152-770	512	495	1080	4.87	800
274	111-431	359	316	1188	4.77	630
330	123-450	318	198	276	1.19	340
272	114-431	282	274	1478	4.94	780
300	157-250	251	193	258	1.24	260
327	130-368	250	252	425	1.92	310
318	127-352	243	271	571	2.53	660
277	106-329	234	124	230	1.13	320
270	108-352	233	143	126	1.25	360
286	112-294	217	161	191	1.27	490
328	95-243	197	144	250	1.39	340
322	118-223	174	125	231	1.26	180
297	114-200	174	153	179	1.09	260
284	81-238	170	142	143	1.43	200
315	108-197	163	124	251	1.29	300
	Aver.	271	221	478	2.21	454

TABLE IV.

Rabbits Fed Cholesterol for Two Months and Given KI for a Third Month Before Killing.

	Blood Cholesterol mg. per 100 cc.					
		Aver.				
Rabbit No.	Range	Feeding Period	3rd Month	Total Liver Cholesterol, mg.	Cholesterol per 100 gm. Dried Liver, gm.	Weight of Adrenals, mg.
271	131-781	525	527	364	1.78	690
263	129-758	522	521	590	2.77	1020
319	131-704	303	395	536	3.57	700
321	130-476	248	174	238	1.54	290
269	108-275	224	208	382	1.84	710
276	92-385	218	311	384	1.99	840
326	121-284	214	153	262	1.69	230
280	120-250	187	162	239	1.41	320
323	118-258	184	135	216	1.71	220
290	108-333	181	211	234	1.16	300
317	115-208	174	164	263	2.09	670
316	112-302	172	254	742	4.10	600
311	96-191	154	114	237	1.34	500
291	118-203	152	135	216	.80	240
	Aver.	247	259	350	1.99	524

esterol determined by the method used for the blood.[3] The weighing was done as quickly as possible to prevent the dried liver from taking up atmospheric moisture.

Liver Cholesterol. The figures for the liver cholesterol in the 4 groups may be summarized as follows:

Group	Total Aver. mg.	Per 100 gm. dried liver gm.
I	218	1.42
II	821	4.50
III	478	2.21
IV	350	1.99

When no cholesterol was fed (Group I) the total cholesterol in the liver varied widely but was uniformly less than 300 mg. Contrasted to this was the finding that each animal in Group II had more than 300 mg. in the liver after 2 months of cholesterol feeding. The tendency of the liver cholesterol to return rapidly to normal levels when cholesterol feeding is discontinued was strikingly shown in Groups III and IV, indicating an apparent reversibility of the liver lesions at this stage. The difference in averages between the rabbits that did and those that did not receive KI was due to the presence of a few very high figures in Group III, while the number with normal amounts of cholesterol in the liver was the same for the 2 groups.

The independence of the level of the cholesterol in the blood and the total amount in the liver is clearly shown in the tables. A further point of interest is that the liver cholesterol was markedly increased in 3 rabbits with a normal blood cholesterol after feeding, hence not included in the tables. This is in sharp contrast to the occurrence of atheromata which, in our experience at least, never appear without a preceding rise in blood cholesterol.

Adrenal Weights. These varied widely. The average weight of a pair from a control rabbit was 297 mg. After cholesterol feeding for 2 months the average weight was 575 mg., presumably due to an increase in lipoids. A month after cholesterol feeding had been stopped the average weight had fallen to 454 mg., but if KI had been given it was 524 mg. If anything, the iodide inhibits the reduction in adrenal weight that occurs normally when cholesterol feeding is stopped.

Blood Cholesterol. The results may be summarized as follows:

Group		Range of individual averages mg.	Group average mg.
I		100-145	117
II		150-527	250
III	feeding period	163-557	271
	3rd mo. (no KI)	124-495	221
IV	feeding period	152-525	247
	3rd mo. (KI)	114-527	259

The rise in blood cholesterol with cholesterol feeding showed the usual individual variation, but was roughly comparable in the 3 groups fed. During the third month when cholesterol had been stopped, the blood cholesterol began to fall promptly in the rabbits that did not receive KI, while, in contrast, there was an actual rise in the average blood cholesterol for the group given KI.

It is a common experience to find that certain rabbits in a group fed cholesterol fail to develop a hypercholesterolemia. There has been no explanation for this resistance. The high incidence of this in the present series was unusual. Thus, of 62 rabbits fed cholesterol, 17 failed to develop a hypercholesterolemia. A point of possible significance was that 13 of the 17 animals were males, a ratio of 3:1, whereas in the total group of 62 rabbits only 30, or approximately one-half, were males.

Atheromata. Gross aortic lesions were present in 8 of the 15 animals killed after 2 months of cholesterol feeding, in 6 of the 14 that received KI during the third month, and in 11 of the 16 that received no KI. The period of feeding by this method was too short to attach much significance to these results.

Conclusions. (1) The cholesterol content of rabbit liver, increased by cholesterol feeding, tends to return spontaneously to a normal value when the feeding is stopped. KI apparently does not accelerate this fall. (2) The normal decrease in adrenal weights and the prompt fall in the blood cholesterol occurring when cholesterol feeding is stopped are both inhibited by the administration of KI at the conclusion of the feeding period. (3) The high incidence of males among rabbits failing to develop a hypercholesterolemia with cholesterol feeding warrants further study.

9091

The Deterioration of Vitamin D in Aqueous Solutions.

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The vitamins may be generally grouped into two classes, namely: (1) those soluble in fats and (2) those soluble in aqueous solutions. Vitamins A, D, and E are found nearly exclusively in association with fats, and vitamins B and C with substances soluble in water.

The reason for the distribution of vitamins in certain vehicles has not been definitely explained, although it may be readily surmised that specific vitamins probably deteriorate in solutions other than those in which they are found in nature. We have encountered such deterioration of vitamin D when emulsified in water.

Shelling and Tidwell[1] prepared oil-in-water emulsions of viosterol, which were miscible in water and in milk. They suggested that such emulsions offered a means by which larger amounts of vitamin D might be added to milk than was possible to impart by direct irradiation of the milk or by feeding vitamin D preparations to the cow. Subsequent clinical experiments[2] revealed that the *emulsified* oily solutions of vitamin D, when added to milk, were more than 10 times as potent as viosterol in oil, not so treated. Thus, in a study of *134* cases of severe rickets studied by Shelling and Hopper,[3] many of which were partially or entirely refractory to ordinary doses of cod liver oil, at least 20 to 30 drops of viosterol were required to heal the rickets completely in an average time of 3.7 months. On the other hand, with emulsions of viosterol, severe forms of rickets may be healed with as little as 1.5 drops of viosterol (125 Steenbock or 350 International units) in an average time of about 3 months.

In the first few months of the experiment, several rachitic children were given the emulsion and healing took place as expected, but after that time healing occurred very slowly and, in some instances, not at all. When a new emulsion was fed to the latter group, healing took place promptly, as in the first group. Bioassays of the old emulsion, made in November, 1933, revealed the fact that it had lost nearly all its potency on standing in the icebox in brown, tightly stoppered bottles for a period of 5 months. The experiment was repeated with the new emulsions and these too showed progressive deterioration. The emulsion, which originally contained 5% of viosterol 250-D, or 500 International units per gram, assayed only 280 units after 3 weeks, 125 units after 6 weeks, and less than 40 units per gram, after 6 months. Some samples showed no potency at all at the end of this period. The containers were opened from time to time and no effort was made to exclude air. The loss of potency was only slight when the emulsions were kept in sealed glass vials, in which the oxygen was replaced by nitrogen. Bioassay of these emulsions after 4 months' standing showed an average

[1] Shelling, D. H., and Tidwell, H. C., PROC. SOC. EXP. BIOL. AND MED., 1934, **31**, 605.

[2] Shelling, D. H., and Hopper, K. B. (To be published).

[3] Shelling, D. H., and Hopper, K. B., *Bull. Johns Hopkins Hosp.*, 1936, **58**, 137.

of 400 as contrasted with 500 International units per gram initially present. The slight deterioration in this case may have been due to the exposure of the emulsion to the atmosphere, while it was being fed to the rats, or to some other unknown factor.

Some of the emulsions were sent to Dr. Charles E. Bills, of the Research Laboratories, Mead Johnson and Company, Evansville, Indiana, who corroborated these findings.

Similar loss of potency of pure vitamin D in crystalline form was noted by Fuchs and Van Niekerk;[4] and a quantitative difference in the potency between freshly prepared water and milk solutions of Drisdol (vitamin D in propylene glycol), stored for months at a low temperature, was observed by Supplee and his associates.[5]

The exact mechanism of the deterioration of vitamin D in states other than oil is not definitely known. It is highly probable that it is due to the relatively high solubility of oxygen in water, in which case the compound may be oxidized more rapidly than in oil solutions. In this respect the stability of vitamin D differs from that of vitamin A, since Hopkins,[6] Mellanby[7] and McCollum[8] have found the latter to be destroyed by bubbling oxygen through heated butter fat or cod liver oil. In Mellanby's and McCollum's experiments the potency of vitamin D in the cod liver oil was apparently not impaired by this procedure.

The gradual loss of potency of vitamin D in aqueous solutions raised the question as to its stability in canned evaporated milk. Accordingly, we have prepared, with the aid of the Research Staff of a dairy producing canned milk, a vitamin D milk in cans, by emulsifying a known quantity of viosterol with a measured amount of milk. The milk fat from sample cans was assayed from time to time and was found to contain the calculated amount of vitamin D, within the errors of bioassay, and when fed to 6 children with very severe rickets, in amounts ranging from 240 to 350 International units (1 to 1.5 drops of viosterol in oil), the rickets healed promptly and completely within a period of from 3 to 4 months.

Inquiry from the Wisconsin Alumni Research Foundation and from several manufacturers of canned, irradiated milk brought the reply that they found such milk stable, with respect to vitamin D

[4] Fuchs, L., and van Niekerk, J., *Biochem. Z.*, 1935, **272**, 32.

[5] Supplee, G. C., Ansbacher, S., Bender, R. C., and Flanigan, G. E., *J. Biol. Chem.*, 1936, **114**, 95.

[6] Hopkins, G., *Biochem. J.*, 1920, **14**, 725.

[7] Mellanby, E., Spec. Rep. No. 61, Med. Res. Council, London, 1921.

[8] McCollum, E. B., Simmonds, N., Becker, E. J., and Shipley, P. G., *J. Biol. Chem.*, 1922 **52**, 293.

potency, even after standing for more than a year. It is interesting that, although most canned milk is not "vacuum packed", most of the air is removed from it before sealing by the process of warming and condensing, and the removal of most of the oxygen by this means may be responsible for the stability of vitamin D in canned milk.

9092

Toxicity of Chlorazol-fast-Pink in Dog Fish.

J. T. HALSEY.

From the Mt. Desert Marine Biological Laboratory.

Chlorazol-fast-pink, one of the azodyes,[1, 2, 3] has been found an effective anticoagulant *in vivo*. In the effort to avoid clotting in arterial cannulae in some pharmacological studies of the circulation of the dog fish, the author has attempted to use this substance. Huggett and Rowe give its M.L.D. for rabbits as 300 mg. per kilo and Feldberg has found that in dogs 80-100 mg. per kilo in 8% solution given intravenously did not exert any deleterious action on heart or vessels. However, the author in some 20 experiments found that the intravenous injection of as little as 20-30 mg. per kilo of this dye in 3% solution regularly depressed the heart and the blood pressure markedly and that about 100 mg. per kilo was a lethal dose.*

This report is made in the belief that it may be of value to any one contemplating the use of this dye in fishes.

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[1] Parkes and Brambell, *J. Physiol.*, 1932, **74**, 65.

[2] Huggett and Rowe, *ibid.*, 1933, **80**, 95.

[3] Feldberg and Guimaris, *ibid.*, 1936, **86**, 306.

* The preparation used was the same (out of the same vial) which in the Department of Pharmacology at Tulane had been entirely satisfactory when given intravenously to cats and dogs in dosage of from 80 to 100 mg. per kilo.

AUTHORS' INDEX

October, 1936, to January, 1937, inclusive

(The numerals indicate the page)

SUBJECT INDEX

October, 1936, to January, 1937, inclusive

(*The numerals indicate the page*)